STUDIES OF TRADITIONAL CHEESES AND FERMENTED MILKS

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DECLARATION

This submission consists of a selection of 71 publications produced over the period 1975 - 2001, which were drawn from the more complete list of 198 original research papers, review articles and books published over the same period. All the publications accord to the definitions of the University, and those submitted as a component of the dissertation (List of Submitted Publications) are presented in the sequence of the discussion below. The List of Total Publications is presented chronologically.

The publications selected for submission are considered to be significant in terms of the ideas and data presented, and to indicate a logical development of a number of scientific concepts relevant to the title of the dissertation. The individual papers are a selection from refereed scientific journals and trade publications relevant to the dairy industry. Where a book has made, in the candidate's opinion, a significant contribution to the subject of the dissertation, the title and contents pages have been included, but chapters in books and conference proceedings have, in the main, been cited only in the List of Total Publications. The selection of the submitted publications emphasises also the candidate's conviction that the quality of research can be judged by, among other criteria, its relevance to the needs of industry in both developed and less-developed countries.

Significant contributions to the literature in other fields have been excluded from the discussion below, but the titles of the papers - excluding papers based on studies submitted by the candidate for previous degrees - are cited in the List of Total Publications.

Furthermore, I, the undersigned, hereby declare that the work contained in this Dissertation is my own original work and has not previously in its in entirety or in part been submitted for a degree at any University.

Richard K. Robinson

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It is with great pleasure that I acknowledge the help and encouragement that I have received from Professor Trevor Britz throughout the time spent on the preparation of this dissertation. In addition, the informal support of Professor Dave Ledward from the School of Food Biosciences, University of Reading has been much appreciated.

It must be recorded also that, over the years, organisations like FAO, the World Bank and the British Council have sponsored students from the Middle East and elsewhere to work under my supervision, and it has been the enthusiastic co-operation of these students that has ensured that many projects reached completion. Quite naturally, they are cited as co-authors on many of the papers, and their involvement is gratefully acknowledged.

ABSTRACT

One of the curious facts about the food industry is that many of the processes in use today were being practised, in some form or other, by the Roman legions as they marched across Europe and beyond. Certainly they were familiar with the basic techniques of fermentation, and much current research into fermented foods is concerned with understanding the fundamental nature of these traditional processes, and how the individual stages in a particular fermentation can be better controlled.

Recent developments in the dairy industry have tended to reflect this pattern and, over the years, my research group has done much to support the expanding markets for yoghurt and similar fermented milks. Our evaluation of the polysaccharide-producing characteristics of starter cultures, for example, encouraged yoghurt manufacturers to match physical properties to the perceived demands of consumers, and most culture suppliers followed this lead by labelling their products with precise designations as to their potential for imparting viscosity to a retail item.

Similarly, my group was the first to record the unique physical properties of the concentrated yoghurt, *labneh*, (~ 230 g l^{-1} total solids) that had been made for hundreds of years by draining whey from natural yoghurt hanging in a cloth or animal-skin bag. This detailed analysis of the product facilitated the application of ultra-filtration to natural yoghurt to generate a product with a quality that matched traditional labneh and, today, factories in the Middle East, Greece and elsewhere are using modern membrane-filtration plants to satisfy a growing market demand.

Our success in publicising the attractive properties of concentrated yoghurt

encouraged me to devote time to yet another 'historical' concept, namely the apparent 'health benefits' derived by small communities in Eastern Europe from consuming kefir and koumiss. In the West, the flavour and texture of these latter products have never been accepted, but employing similar cultures to produce 'health-promoting' bio-yoghurts opened an entirely new avenue for research. As clinical evidence in support of the prophylactic and therapeutic properties of *Lactobacillus acidophilus* and a species of *Bifidobacterium* became available, so it became apparent that the therapeutic advantage that accompanies the regular ingestion of 'bio-yoghurts' depended on the survival of these microfloras over the stipulated shelf-lives of the retail vehicles. However, no laboratory medium was immediately available for the simultaneous enumeration of *Lb. acidophilus* and *Bifidobacterium* along with the yoghurt cultures, i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus*. Designing such a medium became a priority for one of my students, and, even today, the procedures that he derived are being used by consumer groups that monitor the performance of the major dairy companies in England.

If the improved quality of yoghurts and 'bio-yoghurts' had a major impact on consumer perceptions of fermented milks, the food sector in England gradually became aware of an even more dramatic change in consumer attitudes. Thus twenty years ago, cheese meant 'Cheddar' but, following a 'deluge' of television publicity about the attractions of 'exotic' catering, housewives began demanding mozzarella and mascarpone for lavish desserts, Feta to sprinkle over salads and Halloumi to grill or fry. In turn, exporting countries like Italy, Greece and Cyprus came under intense pressure to increase supplies of top quality products. Local manufacturers soon

realised, however, that there was little information available concerning the scientific basis to the procedures employed to make some of these traditional cheeses, and my research group was selected by Funding Agencies in Greece and Cyprus to act as a focus for a series of studies of Feta and Halloumi cheese.

The need to eliminate pathogens from the storage brines of Feta cheese without killing the yeasts and bacteria associated with maturation became an important consideration for exporters, and one of my students exploited a novel procedure employing furocoumarins and long-wave ultra-violet light to achieve the desired selective inactivation. At present, the economics of commercial application are somewhat dubious but, as soon as cheap, synthetic, non-toxic furocoumarins become more readily available, the system may well merit re-evaluation. We did confirm, however, that the metabolic activities of the yeasts and bacteria typically isolated from storage brines are essential for flavour development in Feta cheese, and that similar microfloras are instrumental in the development of the important charactistics of traditional Halloumi cheese. In particular, a new species of lactic acid bacterium, *Lactobacillus cypricasei*, was isolated from samples of the traditional ovine cheese, but whether or not the species has a unique role(s) in the maturation process remains an open question.

Clearly there is still much to learn but, if the activities of my reseach group have added just a little to the scientific background essential for future studies of cheese and fermented milks, then their completion will have been worthwhile.

OPSOMMING

Een van die merkwaardigste feite omtrent die voedselindustrie is dat baie van die prosesse wat vandag gebruik word, in een of ander vorm deur die Romeinse magte gebruik is toe hulle deur Europa marsjeer het. Basiese fermentasie tegnieke was aan hulle bekend, en heelwat huidige navorsing oor gefermenteerde voedsel is gemik daarop om die fundamentele natuur van hierdie tradisionele prosesse te verstaan en hoe die individuele stappe in 'n spesifieke fermentasie beter beheer kan word.

Onlangse ontwikkelinge in die suiwelindustrie reflekteer hierdie patroon, en my navorsingsgroep het oor die jare heelwat gedoen om die groeiende markte vir joghurt en soortgelyke gefermenteerde melk te ondersteun. Ons evaluasie van die polisakkaried-produserende eienskappe van suursels het byvoorbeeld joghurtvervaardigers gehelp om fisieke eienskappe daar te stel wat verbruikers tevrede sal stel. Meeste verskaffers van kulture het hierdie voorbeeld gevolg deur hul produkte so te etiketteer dat duidelik gewys word watter potensiaal dit het om viskositeit aan die finale produk te verleen.

Verder was my groep die eerste om die unieke fisiese eienskappe van die gekonsentreerde joghurt, *labneh* (230 g l^{-1} totale vastestowwe) te bepaal, wat vir honderde jare gemaak is deur die wei van natuurlike joghurt te dreineer deur dit in 'n materiaal- of diervelsak te hang. 'n Gedetailleerde analise van hierdie tradisionele produk het bygedra tot die gebruik van ultrafiltrasie op natuurlike joghurt om 'n produk te gee met dieselfde kwaliteit as tradisionele labneh. Vandag gebruik fabrieke in die Midde Ooste, Griekeland en elders moderne membraan-filtrasie aanlegte om in die groeiende vraag na die produk

te voorsien.

Ons sukses met die bekendmaking van die aantreklike eienskappe van gekonsentreerde joghurt het my aangespoor om tyd te spandeer aan nog 'n sogenaamde "historiese" konsep, naamlik die skynbare gesondheidsvoordele van klein gemeenskappe in Oos-Europa wat kefir en koumiss verbruik. In die Weste is die smaak en tekstuur van hierdie produkte nooit werklik aanvaar nie, maar om soortgelyke kulture te gebruik om "gesondheidsbevorderende" bio-joghurt te produseer, het 'n hele nuwe navorsingsveld daargestel. Soos kliniese bewyse van die terapeutiese en voorkomende voordele van *Lactobacillus acidophilus* en 'n spesie van *Bifidobacterium* bekend gemaak is, het dit duidelik geword dat die terapeutiese voordele wat saamgaan met die gereelde inname van "bio-joghurts", afhang van die oorlewing van hierdie mikroflora oor die gestipuleerde rakleeftyd van die kommersiële produkte. Geen laboratorium medium was egter onmiddellik beskikbaar vir die gelyktydige telling van *Lb. acidophilus* en *Bifidobacterium* tesame met die joghurt kulture *Streptococcus thermophilus* en *Lb. delbrueckii* sub-sp. *bulgaricus*. Die ontwikkeling van so 'n medium het een van my studente se prioriteit geword, en selfs vandag word die prosedures wat deur hom ontwikkel is, gebruik deur verbruikersgroepe wat die optrede van groot suiwelmaatskappye in Engeland monitor. Indien die verbeterde kwaliteit van joghurts en bio-joghurts 'n groot impak gehad het op verbruikers se persepsie van gefermenteerde melk oor Wes-Europa heen, het die voedselsektor in Engeland bewus geraak van selfs 'n meer dramatiese verandering in verbruikers se houding. Twintig jaar terug het kaas "Cheddar" beteken, maar na 'n stortvloed televisie advertensies oor die aanloklikheid van eksotiese geregte, het daar by huisvroue 'n vraag ontstaan na Mozzarella en Mascarpone vir nageregte, Feta oor slaai

en Halloumi om te bak of te braai. Italië, Griekeland en Siprus wat hierdie produkte uitgevoer het, het onder kwaai druk gekom om groter hoeveelhede, top-kwaliteit produkte te lewer. Plaaslike vervaardigers het gou agtergekom dat min inligting beskikbaar was oor die wetenskaplike basis van die prosedures wat gebruik word om hierdie tradisionele kase te maak en my navorsingsgroep is deur befondsingsagentskappe in Griekeland en Siprus genader om studies te doen oor sekere aspekte van die vervaardiging van Feta en Halloumi kaas.

Dit het vir beide in- en uitvoerders belangrik geword om die patogene te elimineer uit die soutoplossing waarin Fetakaas gestoor word, sonder om die giste en bakterieë wat rypwording aanhelp, te dood. Een van my studente het 'n innoverende prosedure ontwikkel wat furocoumarins en lang-golf-ultra-violet lig gebruik om selektiewe inaktivering te kry. Op die oomblik is daar effense onsekerheid oor die ekonomiese implikasies van die kommersiële toepassing, maar sodra goedkoop, sintetiese, nie-toksiese furocoumarins geredelik beskikbaar word, moet die sisteem weer geëvalueer word. Ons het egter bevestig dat die metaboliese aktiwiteite van die giste en bakterieë in die stooroplossing noodsaaklik is vir geurontwikkeling in Feta kaas en dat soortgelyke mikrofloras instrumenteel is in die ontwikkeling van die belangrike karaktereienskappe van tradisionele Halloumi kaas. 'n Unieke melksuurbakterium, *Lactobacillus cypricasei*, is uit monsters tradisionele skaap Halloumi geïsoleer, maar of hierdie spesie 'n unieke rol speel in die verouderingsproses is nog 'n ope vraag.

Duidelik is daar nog baie om te leer, maar indien my navorsingsgroep se aktiwiteite slegs 'n klein bydrae gemaak het tot die wetenskaplike agtergrond wat essensieel is vir toekomstige navorsing, was die voltooiing daarvan die moeite werd.

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Chapter 1. INTRODUCTION

Exactly what evolutionary pressures lead to milk becoming the universal food for infant mammals is a matter for speculation but, for thousands of years, Man has derived immense benefits from this unique characteristic. In particular, the ability of Man to husband large herds of reindeer, cows, sheep, goats or camels has meant that most communities across the world devised various practices to collect milk and utilise it as a component of their normal diets. The same peoples would have been aware also that milk was a very fragile product, and even societies in the cold climate of Northern Europe would have been conscious that liquid milk could become unusable in a matter of hours.

Obviously such societies were not aware that bacteriological spoilage was to blame, but what they would have observed was that the nature of the deterioration was not uniform, and that while some lots of surplus milk became usable only as pig food, others changed in character but remained totally palatible. Thus, the frequent storage of raw milk in a vessel of earthenware or animal skin could well, at some point in time, have lead to the selection of a fermentative microflora, and the repetitive use of the same vessel might well have given rise to a 'sour milk' that had a pleasant flavour and aroma. It would then have been a simple matter for the practice to spread to neighbouring communities, and for a specific 'sour milk' to become a normal part of the regional diet. Evidence in favour of this idea is provided by the nature of the milks themselves for, in Northern Europe, the cool climate lead to the dominance of

mesophilic microfloras and one distinctive range of products, while in the Middle East, thermophilic microfloras emerged to give fermented milks that were totally different in character.

In addition to this natural evolution, Man intervened to improve the sensory properties of the end-product. Partial concentration of the milk over an open fire would have been found to greatly improve the consistency of 'yoghurt-like' products and, in this way, standardised procedures for making these popular foods would have emerged. It is likely, of course, that the quality of the products ranged from excellent to almost inedible, for even at the beginning of the 20th. century, little was known about the bacteria that formed the essential microfloras. Even after the publication of the book by Metchnikoff in 1910 linking longevity among the hill tribes of Bulgaria with the consumption of yoghurt containing special bacteria, the ensuing interest in the 'health-giving' properties of yoghurt did nothing to improve the yoghurt making process. Indeed, the quality of the fermented milks available in towns and villages around Europe appears to have been so poor that even the alleged mystical properties of fermented milks could not persuade consumers to eat them.

1.1 Products Manufactured with Thermophilic Cultures

While fermented milk drinks were developed by producers in Scandinavia and North America, more viscous or 'gelled' products became the popular forms of fermented milk in Southern Europe, Asia and the Middle East. Even allowing for occasional batches of poor quality, yoghurt and similar products easily retained a place in the diet of communities with a long history of consumption, but it was not until the

1950's that yoghurt made any real impact on the commercial markets of Europe and North America. In the event, it was the addition of fruit that proved the turning-point, and fruit yoghurts began to find favour as pleasant 'snacks' for picnics or similar occasions. Control over the fermentation improved also, so that a consumer could purchase products on a regular basis and know, with some degree of confidence, that the flavour and consistency would match his/her expectations. Today, the retail value of the fermented milk market is worth millions of dollars (US) and, with sales continuing to surge, the image of fermented milks as safe, healthy and satisfying 'snacks' has become deeply entrenched. The nature of the retail products has, of course, changed to meet consumer demands for more exotic fruit flavours and/or more satifying textures, but it is interesting that the essential characteristics of many fermented milks have not really altered.

Thus, yoghurt is still defined as a product manufactured from milk - with or without the addition of some natural derivative of milk, such as milk powder or cream - and with the gel structure being the result of coagulation of the milk proteins by lactic acid secreted by defined species of bacteria. Furthermore, these same bacteria, usually *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus* must be 'viable and abundant' at the time of consumption, and a definition along these lines is enshrined in the Food Laws of many countries. Indeed, in many countries, retail cartons may only be labelled as 'yoghurt' if the product contains viable cells of *Lb. delbrueckii* sub-sp. *bulgaricus*, and this protection of the name has ensured retention of the essential characteristics of the product. Obviously, gelled products can be made in other ways and/or the bacteria killed by heat treatments, but such products

should never be confused with a true fermented milk.

Despite the popularity of both natural and fruit yoghurts, manufacturers are always seeking new markets, and the development of so-called 'health-promoting' or 'bio-yoghurts' has, over the last ten years, transformed the supermarket shelves right across Europe. These yoghurt-like products may contain a range of cultures, such as *Lb. acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri* or *Lactobacillus paracasei*, along with one or more members of another genus, *Bifidobacterium*, for example *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium infantis* or *Bifidobacterium lactis*. The most popular culture for such 'bio-yoghurts' contains a mixed flora of *Lb. acidophilus*, *Bifidobacterium* sp. and *Str. thermophilus* and, while the former pair provide the main 'health benefits', the latter is able to acidify the milk rapidly and provide the flavour and physical characteristics expected by the consumer of a yoghurt-like product.

However, although the production of yoghurt is a multi-million dollar industry, it should not be forgotten that the original fermentation process was one of preservation of the valuable nutrients in milk. In reality, of course, even yoghurt spoils rapidly at ambient temperatures above 10°C and, under primitive conditions, this deterioration must have been extremely rapid. Consequently, a number of important derivatives of yoghurt made an appearance. For example, our ancestors in the Middle East may well have observed that, when yoghurt was left in an animal-skin bag and forgotten for a few days, the drainage of whey gave rise to a concentrated yoghurt (labneh) which they could consume over several days with little change in

flavour. They would have discovered also that if some of the labneh was allowed to dry partially in the sun before being placed in a jar of olive oil (labneh anabaris), consumption of the same batch could continue for weeks or even months. Whether or not one of the dietary uses of labneh included mixing with cooked cereal grains is not known, but certainly some tribes in Iraq discovered that, when labneh was blended with ground, boiled wheat and portions of the mixture were dried in the sun, the resultant product (kishk) had a storage life of several years. Obviously reconstituted kishk has few of the properties of the original labneh, but at least the nutritionally valuable milk proteins had not been wasted.

Today, the origins of these traditional products have long been forgotten, and yet 'labneh' is now marketed all over the world labelled as 'strained' or 'Greek-style' yoghurt.

1.2 Products Manufactured with Mesophilic Cultures

Mesophilic species of bacterium like *Lactococcus lactis* sub-sp. *lactis*, *Lactococcus lactis* sub-sp. *cremoris* or *Leuconostoc mesenteroides* sub-sp. *cremoris* that grow at temperatures of 20 - 22°C have long been associated with the cheeses and fermented milks of Northern Europe. In these cooler climates, some form of 'crude' starter culture became almost essential but, in Southern Europe and North Africa, the natural mesophilic microfloras of the raw milk seem to have been sufficent to provide both the acidity for the coagulant to act during cheesemaking and, perhaps, some of the flavour characteristics desired in the product. Cheeses like Domiati, Feta and Halloumi are all made without starter cultures, and it is notable also that all three of

these 'warm-climate' cheeses are matured in brine. The potential preservative action of the brine would have been well known to the early producers, but more recently, it has become evident that the microflora of the brine is essential for flavour development in these same cheeses.

1.3 Aims of the Study

Although fermented foods and beverages have been produced for thousands of years, it was not until the 1950's that the attractions of fermentation as a means of preservation, biological ennoblement and flavour enhancement suddenly became 'fashionable'. What became evident also was that any commercial exploitation of traditional fermentations depended upon acquiring a detailed knowledge of the basic microbiology of the processes, and there have been some notable success stories in this respect. The expansion of the pickled cucumber industry in the USA owes much to the scientists who studied the microbiology of the traditional process, and the commercial market for sorghum beer in South Africa could not have been exploited if the native process had not been modified and standardised.

In the dairy industry, the parallel changes have tended to be less dramatic for, at the end of the day, it must be admitted that yoghurt, for example, is still made by a process derived thousands of years ago. Nevertheless, success of the fermented milk sector has depended on standardisation of the traditional processes, and many of the studies highlighted in this dissertation tend to reflect how fermented milks have evolved over the last thirty years.

Thus, our work on the impact of extra-cellular polysaccharide-producing starter

cultures on the organoleptic properties of yoghurt sought to reveal for manufacturers and culture suppliers alike the potential for modifying consistency to suit consumer expectations, and similar studies on the concentrated yoghurt from the Middle East (labneh) paved the way for its commercial exploitation. In particular, one of the targets for this latter study was to match the properties of traditional labneh made by gravity drainage of the whey with a product concentrated by ultrafiltration, and it may be no coincidence that all the 'Greek-style' yoghurt sold in Europe is maufactured by membrane processing.

In parallel with these studies, two of my students focussed their attention on the prophylactic/therapeutic cultures that were being widely praised for their 'health-promoting' benefits. However, it was widely reported that the potential advantages for the consumer depended on the species being of human origin and present in a product at counts exceeding 1.0×10^6 colony-forming units (cfu) ml^{-1}. Consequently, methods for enumerating *Lb. acidophilus* and *Bifidobacterium* sp. in dairy products were needed urgently for, while the medical profession were familiar with the isolation of *Bifidobacterium* sp. from faeces and other materials, the enumeration of the same genus in a milk product containing, perhaps, three other species of lactic acid bacteria posed a very different problem. Similarly, factors influencing the survival of these probiotic species in milk was poorly understood, for a 'bio-yoghurt' is a very different environment from their normal habitat in the intestine of a human. Although some of this work involved refinement of existing procedures, its real value became linked with consumer protection, and a number of organisations in the UK were concerned to ensure that retail products contained 'therapeutic minimum counts' at the time of

consumption.

If improving the quality of yoghurt and 'bio-yoghurt' has essentially been a process of refining well-documented technologies, the procedures for making Nabulsi cheese from Jordan or Bafut cheese from the Cameroon had, by contrast, never previously been studied in any systematic fashion. Consequently, we instigated a number of studies of these two cheeses that revealed not only their chemical, physical and organoleptic characteristics, but also allowed us to develop variants based on recombined and filled milks. This latter study was of especial relevance to the Cameroon which has an abundance of refined palm oil and easy access to skimmed milk powder from Aid Organisations.

Additional members of my research group extended this work to cover the traditional, brined cheeses of the Mediterranean and North Africa which, in many cases, are still being made from raw milk by small producers using skills handed-down from previous generations. Even with commercial cheeses like Feta and Halloumi, it emerged that the basic microbiology was poorly understood, and yet the market for these cheeses is growing fast. Establishing a baseline of knowledge to assist with this expansion became a major programme in its own right. Thus, while it had long been realised that the microfloras of the cheese brines were important for flavour development, there was a clear need to isolate and characterise the principal species from within these microfloras, and determine their precise biochemical contributions to the maturation process. Indeed, these studies may well help to make it possible to control the organoleptic properties of a cheese like Feta by means of cultures added to the brine.

If Cameroon has the milk or other ingredients for cheesemaking but little expertise, the situation in the Sudan was, at least in the early 1980's, almost the reverse. The local cheese, Gibna Baida, was an excellent product but of limited availability, and one of my students sought to develop a process for the producing an identical cheese by the direct recombination of skim-milk powder and butteroil. As far as I know, this project was the first to exploit the potential of direct recombination for cheesemaking but, sadly, the economic conditions in Sudan deteriorated before the results of the work could be transferred to the 'factory floor'. However, it is pleasing to record that two private companies supported by the New Zealand Dairy Board are employing procedures based on this work to manufacture Feta-style cheeses in the United Arab Emirates, so clearly our publications did not go unnoticed.

As all these ventures were completed on a University campus and often involved young scientists at the start of their careers, the results have always been placed in the public domain. Consequently, their impact on the dairy industry in the UK or elsewhere will never be known for certain, but what these studies tried to achieve should become clear in the following chapters.

Chapter 2. THE PROPERTIES OF YOGHURT

Yoghurt and similar fermented milks have been produced in the warmer regions around the Mediterranean for centuries, but the more widespread popularity of the product throughout Europe, North America and elsewhere is comparatively recent. These latter markets are dominated by two types of retail product. One variant has a firm, gel-like structure together with a clean, mildly acidic and slightly aromatic flavour - *natural set yoghurt,* while the other has the consistency of 'double cream' and the background flavour of yoghurt is usually modified by the addition of fruit/ flavours and sugar (sucrose) - *stirred yoghurt*. Nevertheless, despite the apparently contrasted nature of the end products, the manufacturing procedures for both variants have much in common and, even today, closely resemble the traditional fermentation.

However, the current generation of consumers are more demanding than their ancestors, and not only do they anticipate that an extensive range of products will be available, but that the characteristics of the product will not vary from batch to batch. Modern process controls make it possible for manufacturers to meet these expectations, and this aspect of the study has been concerned with an examination of those facets of the fermentation that are most amenable to standardisation.

2.1 Method of Manufacture

The raw material for yoghurt production is usually bovine milk, although the milk from other mammals, such as the sheep or buffalo, is equally amenable to fermentation; only in camel's milk are the micelles too small to form a proper

coagulum (Ali and Robinson - 1). Caprine milk can also be employed, but, due to the high level of ß-casein and the poor functional properties of caprine α_{s1} casein, we found that the coagulum formed during the fermentation stage was soft and the end-product lacked the attractive, 'mouth feel' of normal yoghurt (Robinson and Vlahopoulou - 2). Although milk fat can be present or absent according to taste and/or market demand, the critical feature of the milk, in the present context, is the level of solids-non-fat (SNF). In bovine milk, the level is 85 - 90 g l^{-1}, of which around 45 g l^{-1} is lactose, 33 g l^{-1} protein (26 g l^{-1} casein and 7.0 g l^{-1} whey proteins) and 7.0 g l^{-1} mineral salts (Tamime and Robinson - 3). Each of these components is vital for the production of a satisfactory yoghurt, in that while the lactose provides an energy source for the starter bacteria (see later), the protein, together with minerals like calcium and phosphorus, gives rise to the basic structure of the gel. However, the levels present in liquid milk are not sufficient to produce a satisfactory end-product, and hence the first step in manufacture is to check the quality of the ex-farm milk and then raise the level of SNF.

2.1.1 Liquid Milk

The basic ingredient of most yoghurt is whole milk or skim milk, and hence the quality of the incoming milk is an important consideration. The methods of extracting representative samples will vary with the size of factory concerned, but it is essential that the portion examined truly reflects the quality of the whole silo or tanker. The availability of automated techniques means that the chemical composition of the incoming milk can be monitored quite easily, but one **essential** test must be for

inhibitory substances. Thus, while minor variations in chemical composition may alter the quality of the end-product and/or the economics of the process, the presence of antibiotics in the milk can lead to total vat failure.

2.1.1.1 Inhibitors of Starter Activity

The production of liquid bulk starter cultures in a cheese or yoghurt factory can encounter problems with respect to: (a) contamination by adventitious bacteria, yeasts or moulds or, possibly, bacteriophage; (b) changes in the activity of the culture, e.g. rate of acid production or level of aroma/flavour compounds secreted; and (c) the presence of antibiotics or other inhibitory substances in the milk.

In particular, *Str. thermophilus* is especially sensitive to antibiotics like penicillin, streptomycin, neomycin and ampicillin which are widely used to control mastitis (Yamani, Haddadin, Al-Kurdi and Robinson - 4) and, when grown alone, contamination of the milk as low as 0.004 International Units (IU) ml^{-1} of penicillin G can inhibit cell wall development; strains of *Lb. delbrueckii* sub-sp. *bulgaricus* tend to be a little more tolerant (0.02 IU ml^{-1} of penicillin G). However, even when the two organisms are growing together under optimum conditions, 0.01 IU ml^{-1} of penicillin G can delay fermentation. Sanitising agents employed to clean a plant, such as chlorine (100 mg l^{-1}) or iodophors (60 mg l^{-1}), can also cause inhibition of the mixed cultures (Tamime and Robinson - 3), and hence the screening of bulk milk for microbiocidal agents is essential.

The Disc Assay is able to detect 0.005 IU of penicillin G ml^{-1} of milk, while the more user-friendly Delvotest P can detect 0.004 IU of penicillin G ml^{-1} of milk

in 2.5 hours; at a level of 0.006 IU of penicillin G ml^{-1} of milk, the Delvotest is reported to be 100% accurate. More recently, the Lac-Tek and Delvo-X-Press tests have been introduced, and these systems can identify a range of ß-lactam antibiotics at levels of 0.006 IU of antibiotic ml^{-1} of milk but, in this case, the detection time is around 7 minutes. This rapid response means that **all** milk required for processing can be tested ahead of its introduction into the production area. The Charm Test(s) offers another alternative for checking for ß-lactam residues, and Standard Methods are cited also for the HPLC detection of sulphamethazine, the Brilliant Black Reduction Test for inhibitory substances, as well as various ELISA techniques for detecting low levels of ß-lactam antibiotics. However, these Standard Methods can prove costly, and one of my students working in Jordan found that even quite simple procedures can, if standardised, give excellent results (Yamani, Haddadin, Al-Kurdi and Robinson - 5).

2.1.2 Fortification of the Milk

Traditionally, the solids content of milk was raised by gently heating the milk in an open pan over a fire, and evaporation of the water was sufficient to achieve the desired effect (Tamime and Robinson - 3). Nowadays, although concentration by evaporation under vacuum or ultra-filtration is practised in large dairies, the addition of whole or skim-milk powder to liquid milk and/or the inclusion of whey or casein-based powders remains the popular choice with small dairies. The choice of one method of fortification over another is based mainly on the availability of process plant, but we did find that the system selected could influence the quality of the final product (Lankes, Ozer and Robinson - 6). However, so long as the process milk has

around 40 - 50 g l^{-1} protein and a total solids of 130 - 140 g l^{-1}, it will provide a suitable base for most commercial fermented milks; for some niche markets for natural yoghurt, total solids levels of 160 - 180 g l^{-1} may be used. The fat content tends to be adjusted according to the demands of the retail market, i.e. 5.0 g l^{-1} for the diet-conscious.

At some locations, skim milk or full-cream milk powder may be the only feasible raw material, but whatever the precise type of powder, an examination of each consignment to ensure its adherence to agreed specifications can avoid problems at a later stage. The yoghurt manufacturer is fortunate, however, in that the process milk does receive a severe heat-treatment, e.g. 85°C for 30 minutes or equivalent, and hence some latitude in respect of the microbiological quality of the milk powder can be tolerated. The same margin of freedom applies to the stabilisers or other ingredients added prior to heating, but materials incorporated into the finished yoghurt, e.g. fruit and flavouring/colouring agents, need to be monitored with particular care.

Although natural yoghurt is based entirely on milk, some countries permit the use of stabilisers to achieve an acceptable consistency with stirred yoghurts. Many of them are complex carbohydrates, and the incorporation of starch/modified starch or one of the plant gums like guar or locust bean at a level of 5.0 - 7.5 g l^{-1} will give a smooth texture to a yoghurt without the need for high levels of milk solids. Thus, guar gum, locust-bean gum, as well as the carrageenans and cellulose derivatives, are long-chain polysaccharides composed of regular arrangements of monosaccharide units, and it is this structure that gives them the ability to bind water. It is significant

also that these complex molecules cannot be attacked by digestive enzymes in the human body, and hence they may help to prevent some types of colonic malfunction by: (a) providing a "bulking agent" for the contents of the intestine; (b) stimulating intestinal peristalsis; and (c) encouraging the growth of desirable microfloras in the colon. Inulin is one short- chain polysaccharide that is of especial interest in this latter respect, and Robinson (7) showed that it also imparted an attractive viscosity to stirred yoghurt.

In addition, guar gum can slow the rate of post-prandial glucose release into the blood stream of humans (Robinson - 8)

2.1.3 Heat-treatment of the Milk

Once the desired level of SNF has been achieved, the milk may be homogenized to ensure both full incorporation of any dry ingredients, and to break-down the fat globules to a uniform size of around 1 μm. This size reduction is essential to prevent the separation of cream during the production of full-fat set yoghurts, but it also improves the consistency of stirred yoghurts.

The next stage involves passing the milk through a plate heat exchanger in order to raise the temperature to 90 - 95°C with a residence time in the holding tube of 7 - 10 minutes. Alternatively, the milk may be heated in the main process vessel to 80 - 85°C, and then held for 30 minutes. The choice of treatment depends on the sophistication of the available plant, but the critical feature is the holding time, for the application of high temperatures alone, e.g. Ultra-High Temperature (UHT) treatments at 140°C for around 2 seconds, does not give rise to an attractive yoghurt

(Tamime and Robinson - 9).

2.1.4 Choice of Starter Cultures

Once the heat treatment has been completed, the milk is cooled to 42°C prior to inoculation with a culture composed of equal numbers of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus*. The technology for handling these cultures can vary but, currently, the most popular routes are: (a) production of a liquid 'bulk starter' using 'sterile milk' - usually made from reconstituted skim-milk powder - and a freeze-dried or frozen culture purchased from a supplier; the process milk will be inoculated at a rate of 20 g l^{-1} with the pre-fermented bulk starter (pH around 5.0); or (b) the purchase of a concentrated frozen or freeze-dried culture that can be added direct to the process milk (Robinson - 10, 11). For sheer convenience, the latter route is becoming more popular.

However, whatever type of culture containing *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* is employed, it is recommended that the two organisms should be present in the ratio of 1 : 1 (chain : chain). In practice, this requirement means checking the balance by direct microscopic examination and, if required, the count can be made quantitative as well, i.e. with a Breed Smear technique. If the number of bacteria is too high to be counted directly, then a 10^{-1} dilution in quarter-strength Ringer's solution can be made prior to preparation of the slides. If the sample is agitated for 30 seconds before the 0.01 ml aliquot is removed, then the microscope fields should contain countable numbers of bacteria.

Staining with Newman's stain, or after defatting, with methylene blue or

Gram's stain is a useful aid to differentiation and, for routine purposes, the number of fields to be examined can be reduced from the figure required, in theory, to give an accurate count. Thus, counting ten fields in a five by five cross-pattern overcomes uneven spreading, and a reasonable estimate of the cell count ml^{-1} of a starter culture can be obtained. The only adjustment required is in relation to the expected ratio, because the chains of streptococci tend to breakdown into small units of two or three cells during dilution. If each one of these units is recorded as "one", then the ratio of streptococci : lactobacilli rises to around 2.7 : 1, and this ratio has been found to be repeatable with cultures incubated at 42°C; this work was reported in some detail by Tamime and Robinson (3).

An alternative technique for obtaining information about the ratio between the two organisms in a starter culture, or in the retail product for that matter, is the total colony count using a medium that selects for one or other species, or differentiates between them on the same plate. The fact that dilution and plating will have broken most of the chains necessitates a modification of the expected ratio, and ratios of 10 : 1 (*Streptococcus* : *Lactobacillus*) may well become the accepted "norm"; the chains of streptococci counted as "one" in the "clump count" tend to be longer than the chains of lactobacilli (Robinson - 12). However, it is important that different strains of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* may behave differently on the same medium.

The performance of yoghurt cultures on Lee's medium is a case in point, and one of my students found that while some strains of *Lb. delbrueckii* sub-sp. *bulgaricus* give white colonies, others produce yellow colonies that are identical to those of *Str.*

thermophilus (Ghoddusi and Robinson - 13). The acid-producing capacity of *Lb. delbrueckii* sub-sp. *bulgaricus* appears to be the critical factor; L-S Differential Agar and Modified Lactic Agar are other media that gave different responses according to the strains of bacteria under examination.

Tryptose Proteose Peptone Yeast (TPPY) Agar with Eriochrome Black gives good differentiation, as does Reinforced Clostridial Prussian Blue (RCPB) Agar and, on both of these media, recovery (following confirmation by Gram staining of selected colonies) is good; extremely clear definition was achieved by incoporating Prussian Blue into TPPY Agar in place of Eriochrome Black T (Ghoddusi and Robinson - 13). If precise counts are needed, it should be noted that recovery from the same culture on different media does, on occasions, differ by a factor of ten.

While a single differentiating medium may be preferred for visual counts, the introduction of automatic colony counters may necessitate a change to the use of a medium selective for only one species, e.g. M17 Agar for *Str. thermophilus* and Acidified MRS Agar for *Lb. delbrueckii* sub-sp. *bulgaricus*, or that gives a total colony count for all organisms of starter origin. However, even laser counters are prone to error, e.g. clusters of colonies close to the margin of the Petri dish, and selective media are not always entirely inhibitory to other organisms. For example, we found that Acidified MRS Agar can support the growth of yeasts as well as lactobacilli and, although the difference in colony morphology was evident to the human eye, the electronic system recorded just one total count. This point could be important if the same medium is employed to monitor total viable counts of starter bacteria in a sample of commercial yoghurt (Tamime and Robinson - 3).

2.1.5 Microbiology of the Fermentation

In part, the use of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* is historical in origin, in that they have frequently been isolated from natural yoghurt made by the indigenous tribes of the Middle East. However, there are good reasons for continuing with the tradition for, when growing in milk, the two organisms interact synergistically, and this protoco-operation is based upon the facts that:

- *Str. thermophilus* grows more rapidly than *Lb. delbrueckii* sub-sp. *bulgaricus*, and releases lactic acid, carbon dioxide and formic acid, all of which stimulate the growth and metabolism of the lactobacilli; and
- the proteolytic action of the lactobacilli on the milk proteins releases peptides, while the peptidase activity originating from *Str. thermophilus* makes available amino acids that are essential for further development of both species (Robinson - 12).

The end result of this mutual stimulation is that:

- both species actively metabolise lactose to lactic acid, so that the fermentation is complete within 3 - 4 hours;
- the metabolites liberated by the two species give yoghurt a distinctive flavour, with acetaldehyde at levels up to 40 mg kg^{-1} as the major component (Tamime, Robinson and Chubb - 14). Other compounds of starter origin, such as free fatty acids, amino acids, acetone, diacetyl and keto/hydroxy acids, contribute to the final flavour, but the importance of these compounds with respect to perceived taste and aroma is poorly understood; and
- some strains of the two species can produce appreciable levels of extracellular

polysaccharides, such as glucans or, alternatively, complex polymers with glucose, galactose and rhamnose as the constituent sugars, and the presence of these metabolites enhances considerably the viscosity/consumer appeal of the end-product (Robinson - 15, 16). Thus, whilst some of the polysaccharide forms a layer over the individual bacterial cells, the remainder forms a network that binds the cells and the casein together into a viscous mass; commercial starter manufacturers have available a range of cultures that differ with respect to the types and levels of polysaccharide synthesised (Robinson - 15, 17).

2.1.6 The Fermentation Process

Yoghurt is defined as a cultured milk produced with thermophilic lactic acid bacteria, usually *Str. thermophilus* and *Lb. delbruckii* sub-sp. *bulgaricus*. These starter bacteria, by converting lactose to lactic acid, are responsible for the reduction in zeta potential that leads to destabilisation of the casein micelles and, incidently, the partial dissociation of calcium phosphate associated with the micelles; solubilisation is almost complete at around pH 5.2 - 5.0. As a result of a further decrease in pH, the casein micelles lose their structural integrity and become unstable. After initial contact between the casein micelles, there is a loss of structural entropy which is accompanied by further aggregation and, under quiescent conditions, a three-dimensional heterogeneous casein network forms that is held together by covalent and other protein-protein interactions. The extent of these interactions is determined by both the concentration of, and the physico-chemical state of, the proteins, as well as factors such as pH. The heat treatment applied to the yoghurt milk further affects this basic

process of gel formation, for the usual treatment of 95°C for 7 - 10 minutes results in the denaturation and, subsequently, aggregation of the whey proteins. During this process, a specific interaction between k-casein and ß-lactoglobulin occurs via reduced thiol/disulphide interchange reactions, and these heat-induced protein interactions are essential for the formation of a yoghurt with desirable textural properties.

In commercial practice, many factors influence the physical characteristics of a yoghurt gel and, while the importance of post-incubation conditions, such as in-plant shearing and storage temperature, cannot be underestimated, it is believed that the rheological characteristics of yoghurt are determined mainly by the gelation/fermentation stage.

Once the milk has been inoculated, it will follow one of two routes (see later) to give either set or stirred yoghurt. After 4 - 5 hours, the fermentation will be complete, i.e. the acidity of the milk will have risen to 12 - 14 g l^{-1} lactic acid (around pH 4.2 - 4.3), and the total populations of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* may each exceed 20 x 10^8 (cfu) ml^{-1} (Robinson - 12).

At this acidity, which is probably the level preferred by most consumers, the milk proteins will have coagulated to form a firm gel, and the product must be cooled to avoid over-acidification. If this control is not exercised, then: (a) the product may develop an excessively sharp, sour taste; and (b) the protein gel may begin to shrink and cause whey to separate as a discrete layer on the surface of the yoghurt. This free whey can, of course, be stirred back into the body of the product but, in set yoghurts at least, its presence must be regarded as a fault.

2.1.7 Final Processing

For set yoghurt coagulated in the retail cartons, cooling can be achieved by blowing cold air through the incubation room, or by carefully transferring groups of cartons in their retail trays to a chill room at 2 - 4°C. In-tank cooling of the base is often practiced for stirred yoghurt, and this stage requires the circulation of chilled water (2°C) through the jacket and/or cooling system of the vessel. Care must be exercised at this stage to avoid the formation of 'pips', for these hard pieces of protein can appear as unsightly 'white spots' in coloured yoghurts like the cherry or raspberry varieties. However, while my research group did prove that poor process control could initiate the formation of these small, protein-rich 'lumps', (Robinson - 18), there have been reports that the defect is most prominent during the spring and autumn months. Whether this periodicity is linked with seasonal changes in milk composition has not been established, nor is it clear why :(a) some manufacturers observe the problem more than others; (b) why changes in starter culture can often solve the problem; and (c) why reversion to the original culture after only 2/3 weeks does not lead to re-occurrence of the problem (Tamime and Robinson - 19).

Alternatively, the yoghurt may be stirred and pumped through a plate or tubular cooler, but this process can lead to a loss in viscosity; as most stirred yoghurts are further processed, the initial cooling is usually to around 15°C. In-tank mixing of the fruit or other flavouring mixture (100 - 150 g l^{-1}) can be used, but large factories tend to feed the yoghurt base and the fruit through a blending tube and then directly into cartons. The most popular size of carton, as with the set yoghurt, is the individual portion (125 - 150 g), and the necessary cartons are either: (a) purchased

pre-formed, filled and then heat-sealed with an aluminium foil lid; or (b) the polystyrene or similar material may come as a roll which is formed into cartons on the filling machine - the form/fill/seal process. Family packs (500 g) are available as well, usually with press-on lids to allow for consumption over several days (Tamime and Robinson - 19).

2.2 Quality of the Retail Product

The quality of any food product can be defined against a wide range of criteria including, for example, the chemical, physical, microbiological and nutritional characteristics, or simply in relation to its overall appeal to potential consumers. As a result, quality has to be judged by a range of tests of varying degrees of objectivity, and yet all of them can be useful in ensuring that a product:

- is safe for human consumption with respect to both chemical or microbial contamination;
- conforms to any Regulations enshrined in law, or set down in advisory documents supplied by Public Health or other Authorities/Agencies;
- is capable of achieving a specified shelf-life without spoilage; and
- has a sensory quality that is compatible with existing constraints of manufacture or marketing.

An examination of some of these points implies, naturally enough, that a critical laboratory assessment of the retail product is essential, but it is important to bear in mind that quality control is just one component of the all-embracing concept - Total Quality Management, and this relationship is one that demands constant

attention.

2.2.1 Nutritional Value

(i) Chemical Composition

The increase in SNF will, of course, improve the compositional analysis of yoghurt *vis-a-vis* milk, e.g. > 50 g l^{-1} total protein *versus* 32 g l^{-1} in full-cream milk or > 1.80 g l^{-1} of calcium *versus* 1.15 g l^{-1} in milk; in addition, the bioavailability of the nutrients like calcium and zinc is enhanced as a result of the fermentation. Some increases in B vitamins and folic acid have been recorded as well, but the extent of any increase can vary from product to product - a reflection, perhaps, of strain differences between starter cultures.

Another pertinent characteristic is that the milk proteins in yoghurt are already coagulated prior to ingestion, and the "soft clot" formed in the presence of acid in the stomach may have specific benefits, namely: (a) the softer structure is alleged to reduce any feelings of discomfort; and (b) the more "open" nature of the casein aggregates allows proteolytic enzymes in the alimentary canal easy access during digestion. This latter point may be important for certain vulnerable groups like the young or the elderly, for milk protein can provide a valuable source of essential amino acids.

In addition, it should not be forgotten that milk fat contains an extremely wide range of fatty acids. Most of these are present in the form of glycerides, but over 400 individual fatty acids have been identified in bovine milk. Obviously, it is quite impossible to assign a physiological role to all but a handful of these acids, but the

fact that they are present in a normal mammalian secretion suggests that 'ignorance of function' should not be equated with 'no function'.

Lactose is the principal substrate for the production of lactic acid, and this can be present in yoghurt in two isomeric forms - L(+) and D(-). *Str. thermophilus* produces the L(+) form, while *Lb. delbrueckii* sub-sp. *bulgaricus* releases the D(-) isomer or a racemic mixture DL depending upon the strain. In nutritional terms, the L(+) isomer, which accounts for some 50 - 70% of the total acidity, is the easily digested form while, by contrast, the D(-) isomer is poorly metabolised and excessive intake can cause acidosis in some children.

(ii) Microbiological Aspects

The colon of the adult human contains millions of bacteria *per* gram of content, and some of the constituent species produce a range of phenolic compounds, such as skatole and indole, which can damage living tissue. Indeed, there is concern over their possible involvement in the initiation of cancer in the lower intestine, and hence any dietary component that tends to suppress the production of such compounds could be advantageous. For example, the lactic acid in yoghurt could, by modifying the pH gradient along the intestine, help to inhibit the growth/metabolism of the putrefactive bacteria, but the potential impact is difficult to predict.

Indeed, a possible prophylactic/therapeutic role for yoghurt has been criticised on the grounds that neither *Str. thermophilus* nor *Lb. delbrueckii* sub-sp. *bulgaricus* are likely to survive the digestive process in humans, while the level of lactic acid in the product is relatively small. Nevertheless, it is feasible to suggest that, as the millions of cells of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* autolyse

within the small intestine, so the escaping enzymes and other cell contents could have an effect by stimulating the native microfloras of lactobacilli and bifidobacteria.

Indirect evidence in support of this idea is well documented, for there is no debate about the acceptability of yoghurt (as against milk) in the diet of 'lactose mal-digestors'. These are people who have lost the ability to secrete lactase from the intestinal wall so that, on ingestion, the lactose in milk passes unaltered into the colon. Here it is fermented by the natural microflora, and the gas generated during the fermentation causes extreme discomfort. However, yoghurt is acceptable because the ß-galactosidase released from the degenerating cells of the culture hydrolyses most of the lactose in the small intestine, and hence only insignificant amounts reach the colon. If this enzyme is released from the starter culture and retains its activity during digestion, other cell constituents may behave in the same manner and, perhaps, act as stimulants of the native intestinal microflora.

2.2.2 Considerations of Microbiological Quality

The severe heat treatment received by the process milk, together with the low pH of the final product, makes yoghurt extremely safe in respect to public health, for none of the recognised pathogens can survive or grow below pH 4.3 (Keceli and Robinson - 20). The spores of *Bacillus cereus*, for example, will not germinate at low pH, while organisms of concern in soft cheeses, e.g. *Listeria monocytogenes*, were shown to be inactivated in a low pH yoghurt long before it might reach a consumer (Gohil, Ahmed, Davies and Robinson - 21, 22). In addition, there is good evidence that metabolites from the yoghurt organisms can actively depress the viability of many

enteric pathogens, such as *Campylobacter, Escherichia* or *Salmonella* spp.. Hydrogen peroxide is one such metabolite released by *Lb. delbrueckii* sub-sp. *bulgaricus*, and some strains of the same organism are reported to secrete an antibiotic called bulgarican; *Str. thermophilus* may also release a low molecular weight compound with bacteriocidal properties. However, the activity of these bacteriocins is strictly limited and, in reality, they may do little more than reinforce the effect of acidity (Robinson - 12).

Spoilage, however, can occur through the activities of acid-tolerant yeasts, or occasionally moulds, and widely distributed yeasts, like *Candida* or *Saccharomyces* spp., can be associated with gas formation and/or carton 'doming' of fruit yoghurts (Tamime and Robinson - 19). Excessive acidity, as a result of continued starter activity during prolonged storage above 5°C, can also be a problem, because the acid-tolerant *Lb. delbrueckii* sub-sp. *bulgaricus* has the ability to generate lactic acid to levels of 17 g l^{-1} or even above - depending upon the strain. Such a level is too harsh for the palates of most consumers, and it is this post-production acidification that, in general, tends to determine the shelf-life of commercial yoghurt.

2.2.3 Analysis of Chemical Composition

Many countries have legal standards, or at least provisional regulations, covering the composition of yoghurt. The requirement for a value for 'SNF' is, in reality, more decorative than essential, because the texture or viscosity of a natural yoghurt with an SNF below the usually stipulated minimum would be quite unacceptable. An overall measurement of total solids could, however, be valuable as

a check that the concentration or fortification has been carried out correctly, and a modification of the standard gravimetric method for milk has been proposed as suitable for yoghurt. The routine measurement of protein is essential in large dairies because, over a typical year, the protein content of bovine milk may vary from 32 - 36 g l^{-1}, and these differences are enough to alter both the quality of the yoghurt and the economics of production.

The other significant component, namely fat, is of interest not only in relation to any legal standards, but also because: (a) many stirred yoghurts are designated as low or very low fat, and hence it is important that the description should not be misleading; (b) milk fat has a major impact on the 'mouthfeel' of yoghurt, and around 10 g l^{-1} is regarded as being the minimum to produce the desired response from the consumer; and (c) it is anticipated that full-fat natural yoghurt (30 - 35 g l^{-1}), 'luxury' fruit yoghurts ($>$ 40 g l^{-1}) and Greek-style yoghurts ($>$ 80 - 100 g l^{-1}) will have high fat contents, and again these expectations must be met. The gravimetric methods of determining fat in yoghurt (e.g. the Rose-Gottlieb Method) are regarded as the most accurate but, for routine purposes, the normal Gerber method using 11.3 g of yoghurt in a milk butyrometer is totally appropriate.

The production of lactic acid beyond the point of coagulation is monitored principally in relation to consumer preference, and hence the selected end-point will vary not only from country to country, but also with the type of yoghurt. However, although the relationship between titratable acidity and pH is not straightforward in a highly buffered system like yoghurt, the direct electrometric determination of pH is extremely convenient and more widely used than titration.

2.2.4 Assessment of Physical Characteristics

Yoghurt is normally retailed in one of three physical states, namely as a gelled (set) yoghurt, a viscous (stirred) yoghurt or a fluid (drinking) yoghurt, and each type should have quite distinctive characteristics. However, while the typical gel structure of a set type could never really be mistaken for the semi-fluid form of a stirred variant, the low viscosity of some "stirred" brands leaves the consumer with little option but to drink them. This degeneration of product image is obviously regrettable and, although the release of an occasional "poor" batch is inevitable, the question of "desirable viscosity" is always somewhat vexing. In practice, each manufacturer will probably adopt an agreed "in-house" standard for viscosity (or consistency in the case of set yoghurt), and then operate to this specification, so that the routine assessment of these physical features becomes a normal part of quality control.

(i) Set Yoghurt

The essential gel structure of set yoghurt means that asessment of the product must be approached in a manner that does not destroy the delicate coagulum, and the most convenient test involves the use of the penetrometer. The only special adaptation centres on the choice of spindle and cone, for these have to be selected so that, for the product in question, the depth of penetration of the cone does not exceed about 33% of the total depth of the retail sample. The risk of 'edge effects' from the carton can be minimised by choosing a spindle with a diameter no greater than 50% of the diameter of the pot and, with these restrictions in mind, it becomes a simple matter to select a probe/spindle weight that is appropriate.

In our studies, the effect of the configuration of the probe, i.e. flat or cone-shaped, proved to be a useful variable, so that products with total solids anywhere between 120 and 160 g l^{-1} were handled with ease. In addition, the weight of the probe/spindle was changed in relation to the temperature of the product, e.g. a light spindle for examination of a carton at 42°C immediately after incubation, and a heavier spindle for assessment of the firmer coagulum that developed in yoghurt held at 4°C for 24 hours. These options to change the weight of the spindle made it possible to discriminate, at any given temperature, between samples of different gel strengths, and the fact that comparisons were possible at 42°C made it feasible to predict the consistency of the retail product prior to final cooling; again this work was summarised in Tamime and Robinson (3).

The technique is, therefore, both reliable and versatile, and hence standardising the physical properties of set yoghurt becomes a straightforward exercise. However, if the data are required for research and development purposes, we found that use of a computerised Texture Profile Analyser (TPA) improved the repeatability of the measurements (Ozer, Robinson, Grandison and Bell - 23). Other physical features or faults, for example 'lumpiness' or the presence of 'nodules', usually become apparent during sensory analysis.

(ii) Stirred and fluid yoghurt

The ease of operation makes the rotational viscometer, such as the Brookfield Synchro-Lectric, the popular method for monitoring the viscosity of stirred yoghurts for, once the type of spindle and the optimum speed of rotation have been established for a given product, comparison between successive batches presents few problems;

the Helipath system is preferable as there is less risk of the spindle causing local syneresis and an artificially low reading (Robinson - 15).

However, although methods of this type have the speed and simplicity essential for routine quality control, some authorities argue that the actual figures do not reflect the true nature of the product, in that the shearing effect of the spindle destroys the integrity of the coagulum (Ozer, Robinson, Grandison and Bell - 23). Consequently, it has been suggested that, as stirred yoghurt is a visco-elastic material, i.e. has some of the properties of a viscous liquid and some of an elastic solid, dynamic oscillatory testing would be more appropriate.

Certainly, this non-destructive technique did expose differences in the rheological characteristics of strained yoghurts that would not have been so apparent on the basis of measurements with a viscometer, but the disadvantage of this approach is that: (a) the equipment is expensive in contrast with a viscometer; and (b) taking the measurements can be technically demanding.

2.2.5 Assessment of Organoleptic Characteristics

To some extent, the chemical and physical analyses suggested earlier, e.g. titratable acidity and viscosity, will provide a reasonable indication that the normal "in-house" standards have been achieved, but the use of some form of taste panel to perform a final check is the usual practice. The derivation of a scheme of assessment that can be employed as part of routine quality control will be the first stage and, while the ultimate selection of a scheme will rest with the panel concerned, the over-riding factors must be operational simplicity and the ability of the procedure to

discriminate between samples.

For example, Qualititative Descriptive Analysis (QDA) was applied to natural yoghurts produced with different starter cultures (Robinson - 15). Ten terms were employed to describe the flavour or mouthfeel of the yoghurts, and the scheme was equally applicable to stirred fruit/flavoured yoghurts. Once the terms covering a typical sample had been agreed by a panel and the profile ('spider-diagram') drawn on a transparent sheet, the profiles for subsequent samples could be easily compared by super-imposition. In fact, since the publication of this paper a number of workers have successfully applied QDA to yoghurt and yoghurt-like products.

It is worth noting also that the description of defects can be a valuable part of the exercise, because a quality controller may then be in a position to indicate why the particular batch of yoghurt has scored poorly in certain respects.

2.3 Conclusions

Although the production of yoghurt remains a natural fermentation, the dairy industry has sought over the years to minimise the normal variability of the process. Starter cultures can now be selected on the basis of their contribution to the organoleptic properties of the end-product and control procedures have been devised to monitor all aspects of product quality. A number of these aspects have been studied by my research group over the years, and the results published in the papers cited. In addition, a major overview of then current knowledge of yoghurt was published in 1985 - *Yoghurt:science and technology* (Tamime and Robinson - 3).

Chapter 3. 'HEALTH-PROMOTING' DAIRY PRODUCTS

The potential role(s) of starter cultures in dairy fermentations is to assist in: (a) the preservation of the milk by the generation of lactic acid and antimicrobial compounds; (b) the production of flavour compounds and other metabolites that will endow a product with the organoleptic properties desired by the consumer; (c) the enhancement of nutritional value; and (d) the provision of special therapeutic or prophylactic properties due to the presence, at the time of consumption, of several million, viable bacterial cells of starter origin.

All these activities have, to varying degrees, been discussed earlier, but it is the latter aspect that merits a more detailed appraisal. Thus, while it was nearly one hundred years ago that Metchinkoff suggested that yoghurt could promote a healthy body and additional longevity and the Russians were making similar claims for kefir and koumiss, abuse of the concept rapidly lead to its downfall. In the USA in particular, Acidophilus milk - with *Lb. acidophilus* as the principal bacterium present - was promoted widely as a 'miracle cure', even though most retail samples had few viable cells and little effect on the consumer. It was not suprising, therefore, that widespread enthusiasm for such products evaporated quite quickly.

As happened in so many fields, the advent of the Second World War rescued the situation, for the Japanese discoved that certain fermented milks were an excellent cure for intestinal infections acquired in the jungle. From these primitive origins, Yakult entered the diet of Japanese consumers and, as usage spread across Japan, so clinical studies established that ingesting large numbers of specific lactic acid bacteria could indeed cure patients with certain forms of intestinal ailment. Given the long-

standing acquaintance of North Americans with Acidophilus Milk, interest in this product revived, as did fundamental research focussed on overcoming the losses in cell counts that had destroyed the reputation of earlier generations of the product.

3.1 *Lb. acidophilus* and its Potential

Lb. acidophilus was first isolated from the faeces of bottle-fed infants by Moro in 1900, and was given the name - *Bacillus acidophilus*. Later it was designated as *Lb. acidophilus* and, although this nomenclature is widely accepted, the situation over the status of 'biotypes' remains unclear, i.e. exactly how does an isolate of *Lb. acidophilus* from poultry differ from an isolate of human origin ?. Nevertheless, some members of the medical profession were sufficiently convinced of the possible virtues of ingesting *Lb. acidophilus* as to encourage a modest level of enthusiasm within the dairy industry, and a fairly standard production schedule for Acidophilus Milk evolved.

The reason for this support from doctors stemmed from the fact that *Lb. acidophilus* is a Gram-positive, rod-like bacterium that is a normal inhabitant of the lower end of the small intestine and, in this niche, the species both occupies the lumen of the gut and adheres to the surfaces of the intestinal walls. In this position, the species:

- secretes lactic acid which lowers the pH of the intestinal content and helps to inhibit the development of invasive pathogens like *Salmonella* spp.;
- competes successfully for space and nutrients against pathogenic bacteria, and reduces the time that a post-infection individual can act as a 'carrier'; and

- metabolises any residual lactose in the gut content, so helping to avoid discomfort for consumers with low levels of indigenous lactase (Robinson - 24).

In addition, high population levels may stimulate the immune system of the host and, perhaps, lower the cholesterol content of the blood plasma; at least with sheep, we found that *Lb. acidophilus* lowered the cholesterol level in blood serum (Haddadin, Lubbadeh, Al-Tamimi and Robinson - 25). However, one question still remains: is there any justification for suggesting that products with *Lb. acidophilus* amongst the live microfloras should be placed in the category of 'functional foods', i.e. having special therapeutic properties not related to their chemical/nutritional composition ?

The positive answer appears to be based upon the facts that *Lb. acidophilus* plays a well-defined role in the normal functioning of the human intestine, and that the normal populations levels of *Lb. acidophilus* and related species can be disturbed by abnormal stresses. The excessive consumption of alcohol or onions/garlic can severely deplete the populations of lactobacilli in the small intestine, as can the administration of oral antibiotics. Replacement of the 'missing' microfloras of lactobacilli is one established reaction to the consumption of *Lb. acidophilus* in Acidophilus Milk or a 'bio-yoghurt', as is the regeneration of the indigenous strains. The only reservation is that any therapeutic activity will only be manifest if the number of viable cells is around 2.0 - 3.0 x 10^7 cfu ml^{-1} of product consumed, and that consumption is on a 'regular basis' (Robinson - 24). What is meant by 'regular basis' is an open question, and it will be very difficult to establish any data, under clinical conditions, for 'healthy' humans.

Nevertheless, in spite of these obvious question-marks, the market for products

containing 'health-promoting' cultures has developed rapidly (Robinson - 17). As mentioned already, one of the essential conditions for the success of these products with respect to human health is that the 'therapeutic minimum' number of cells must survive until the product is consumed, and it is important that, in the presence of the mixed cultures that might be used for the production of an 'Acidophilus Yoghurt', survival of *Lb. acidophilus* may be impeded by the antagonism of the other species present. Hydrogen peroxide resulting from the metabolic activity of *Lb. delbrueckii* sub-sp. *bulgaricus*, for example, could have an inhibitory effect, but we did confirm that this potential problem can be avoided by a judicious selection of the yoghurt culture employed (Robinson - 26).

3.2 The Emergence of 'Bio-yoghurts'

While a product like Sweet Acidophilus Milk could find a market in Europe for pouring over breakfast cereals, 'spoonable' yoghurts tend to dominate the fermented milk market in the UK (Robinson - 17). Consequently, manufacturers of therapeutic products have concentrated on the production of so-called 'bio-yoghurts'. Codes of Practice tend to prevent the use of the term 'yoghurt' as *Lb. delbrueckii* sub-sp. *bulgaricus* is not the dominant culture involved, and hence most brands have adopted names like 'B/A - yoghurt' (Bifidus/Acidophilus - yoghurt).

This latter nomenclature also reflects the fact that most manufacturers include high counts of both *Lb. acidophilus* and *Bifidobacterium* spp. in their products, with 'high' meaning above the 'therapeutic minimum' of 1.0×10^5 cfu ml^{-1} for each organism (Robinson - 24). Thus, *Bifidobacterium* spp., as with *Lb. acidophilus*, has a distinct role to play in the ecology of the human intestine, and members of the

genus can withstand passage through the stomach and small intestine when ingested as part of a meal.

3.2.1 *Bifidobacterium* spp. and their Potential

Bifidobacterium spp. are Gram-positive organisms with most distinctive morphologies (Tamime, Marshall and Robinson - 27), which range from Y-shaped to bone-like to coccoid depending on the growing conditions and/or species (Samona and Robinson - 28). A number of species are present in the large intestine, e.g. *Bif. bidium*, *Bif. longum*, *Bif. adolescentis* and *Bif. infantis* and, in this niche, the organisms will:

- secrete lactic and acetic acids which will lower the pH of the colonic content and help to inhibit the development of invasive pathogens like *Escherichia coli* or *Candida* spp.;
- compete successfully for space and nutrients against pathogenic or putrefactive bacteria - it is alleged that amines and phenols released during the metabolism of the latter group could trigger the onset of colon cancer; and
- metabolise the mucin present on the wall of the colon; this essential function not only controls the volume of mucin on the surface of the wall, so avoiding constant diarrhoea, but establishes bifidobacteria as the dominant wall flora (Tamime, Marshall and Robinson - 27).

In addition, high population levels may stimulate the immune system of the host.

3.2.2 Starter Cultures for 'Bio-yoghurts'

While bulk starter cultures are still used for the production of normal yoghurt,

the cultures for 'bio-yoghurts' are usually of the freeze-dried or deep-frozen, direct-to-vat type (Robinson - 10). The reason for this contrast is that: (a) *Lb. acidophilus,* or similar intestinal lactobacilli like *Lb. casei* or *Lb. reuteri*, and *Bifidobacterium* spp. are difficult to grow in milk; and (b) the end-products must have viable cell counts above the agreed 'therapeutic minimum'. By using a direct-to-vat culture with a known cell count, the manufacture is able to calculate with some accuracy the incubation time necessary to obtain the desired final counts and, equally important, can have confidence that those same counts will be achieved day after day.

While some products like the Danish milk, Cultura, contains strains of *Bif. bifidum* and *Lb. acidophilus* alone, most manufacturers seek to improve the flavour and viscosity of 'health-promoting' products through the use of mixed cultures. The employment of yoghurt cultures was the preferred route at one time, particularly as the same culture can, as mentioned earlier, give marked improvements in fermentation times.

However, the high levels of lactic acid generated by normal mixtures of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* when employed alongside the 'bio-cultures' can pose special problems. For example, the counts of bifidobacteria in a 'bio-yoghurt' were shown in our laboratory to decline well below the 'therapeutic minimum' within the anticipated shelf-life of the product (Robinson -29; Samona, Robinson and Marakis - 30). For this reason, *Str. thermophilus* is usually employed alone to generate the acidity desired in a 'bio-yoghurt' for, unlike *Lb. delbrueckii* sub-sp. *bulgaricus*, the production of lactic acid by *Str. thermophilus* is limited to a maximum of around 1.0%. It is relevant also that the production of lactic acid by *Bif. bifidum* and *Lb. acidophilus* in milk is similarly restricted and, at low temperatures

(2 - 4°C), acid production by *Str. thermophilus, Bif. bifidum* and *Lb. acidophilus* ceases almost completely. Consequently, reductions in pH during the storage life of these mixed-culture products are negligible, and the acid-induced decline in viable counts ceases to be a problem.

This restriction has also presented manufacturers with a further benefit for, so long as the pH of the 'bio-yoghurt' is 4.4 - 4.6 after cooling and packaging, the nature of the product does not alter. Consequently, consumers who find the taste of normal yoghurt too acidic can eat 'bio-yoghurts' with few inhibitions, and producers have found that a whole new market has developed - such consumers may not even be aware of the alleged health benefits. However, it must be remembered that, with regard to consumer safety, pH 4.3 is generally accepted as a upper 'safe' limit (Keceli and Robinson - 20), and little is known about the survival of pathogens in 'bio-yoghurts' at pH 4.4 or above.

3.3 Aspects of Quality Control

Acceptable properties like viscosity and flavour are expected of 'bio-yoghurts' just as they are of normal yoghurt, but the essential requirement for high viable counts of *Bifidobacterium* sp. and *Lb. acidophilus* means that additional checks are necessary to ensure that the 'therapeutic minima' are maintained throughout the shelf-life of the product. However, few commercial products contain *Lb. acidophilus* and/or *Bifidobacterium* alone, so that any system for quality control must be able to cope with the presence of *Str. thermophilus* and, perhaps, *Lb. delbrueckii* sub-sp. *bulgaricus* as well.

Consequently, a range of media has evolved for examining 'bio-products' for

Lb. acidophilus when growing alone in a fermented milk, and for *Lb. acidophilus* in the presence of other genera. The choice of medium does, as mentioned elsewhere, depend on: (a) the personal preference of the operator; (b) whether the assessment will involve automatic counting; (c) whether differentiation between a number of species on one plate is an advantage; and (d) the strains of each species, because the 'reactions' of starter cultures on media are often strain-dependent (Ghoddusi and Robinson - 13).

Some media, such as TPPY Agar with Prussian Blue (see page 18), are good for differentiating the normal yoghurt microflora, but offer the additional advantage of enabling an inoculum of a yoghurt culture, bifidobacteria and *Lb. acidophilus* to be enumerated on the one medium (Ghoddusi and Robinson - 13). Thus, colonies of *Str. thermophilus* appear as small, pale blue colonies with a thin, light blue zone in the medium, while *Bifidobacterium* spp. give rise to white colonies, *Lb. delbrueckii* sub-sp. *bulgaricus* produces small, shiny white colonies surrounded by a wide, royal blue zone, and *Lb. acidophilus* is readily distinguished as large, pale blue colonies surrounded by a wide, royal blue zone. It must be emphasised again that differences between strains may prove to be important, and the medium may need further modification and improvement.

If *Bifidobacterium* spp. are the only organisms of interest, then we found that species of human origin can be isolated selectively on a number of media (Samona and Robinson - 31). However, in the presence of other lactic acid bacteria, selective agents have to be employed, and the choice and level of the agents can have a dramatic impact on recovery. Thus, on Blood Liver Agar supplemented with neomycin sulphate, paramomycin sulphate, nalidixic acid and lithium chloride (NPNL)

at a rate of 50 ml of stock solution *per* litre of medium, *Bif. bifidum* showed acceptable recovery while *Bif. longum* was seriously inhibited; the same contrast in growth responses was observed on Modified Rogosa Agar supplemented with NPNL. Successful growth of both species of *Bifidobacterium* was recorded with lithium chloride and sodium propionate as the only inhibitory agents, but unfortunately certain mesophilic and thermophilic lactic acid bacteria were found to grow on the same medium. The most promising option appeared to involve the use of Tryptone Phytone Yeast (TPY) Agar with 20 ml of NPNL *per* litre of medium which gave good recovery for *Bif. bifidum*, *Bif. longum* and *Bif. adolescentis* while, at the same time, eliminating the growth of other thermophilic cultures (Samona and Robinson - 31).

However, the need to check any proposed medium against the strains being employed in the factory was emphasised by the fact that, while *Lact. lactis* sub-sp. *lactis* (NCDO 763) was inhibited on TPY Agar with 20 ml l^{-1} NPNL, *Lact. lactis* sub-sp. *lactis* (NCDO 276) grew; similar patterns could probably be observed with strains of *Str. thermophilus* and *Lb. delbrueckii* sub-sp. *bulgaricus* as well.

Unless colony morphology/colour eliminates confusion, it is important to note that checks on typical colonies under the microscope may not prove helpful. Thus, while the cells of bifidobacteria are usually Y-shaped or bone-shaped depending upon the species, they may become coccoid if the growing conditions so dictate (Samona and Robinson - 28). Consequently, the observation of Gram-positive cocci in a Breed Smear made from a colony on a plate presumed to be selective could, in fact, result from the presence of **either** a normal strain of *Str. thermophilus* **or** an 'abnormal' colony of *Bifidobacterium* sp.. It may be worth mentioning, however, that the change in morphology tends to occur over several generations, so that the examination of a

bio-yoghurt manufactured with a direct-to-vat culture should reveal typical Y-shaped or bone-shaped cells.

In addition, it is important that the concentration of any antibiotic mixture was shown by Samona and Robinson (31) to have an significant impact on the total level of recovery of bifidobacteria and, bearing in mind that 'health claims' should be supported by high cell counts in the product, media selection becomes a vital issue.

3.4 Conclusions

Clearly, there are many attractive 'health-promoting' products on the market and, as mentioned earlier, there may be some justification for suggesting that these products with their live microfloras should be in the category of 'functional foods', i.e. having special therapeutic properties. However, if the positive evidence looks promising, it has to be admitted that there are some negative aspects that must not be forgotten. Excessive intakes, apart from leading to slight intestinal upsets, should not pose a problem, but there must be some concern about:

- poor strain selection - for example, some strains of *Lb. acidophilus* from animals grow better in milk than strains of human origin (Haddadin, Lubbadeh, Al-Tamimi and Robinson - 25), and hence manufacturers might well be tempted to use animal strains for manufacture. A parallel situation exists with another 'health-promoting' genus, *Bifidobacterium*, for *Bif. animalis* - which has never been isolated from humans - is far better suited to the manufacture of fermented milks than the species of human origin. The widespread usage of *Bif. animalis* under the revised name, *Bif. lactis*, is the subject of serious debate and, while *Bif. lactis* may be an entirely appropriate

organism for its intended therapeutic role, the controversy does highlight the problems of strain selection.

- choice of inappropriate products - excessively acidic products will clearly pose a situation that needs to be monitored, but the impact of flavouring or other agents needs careful consideration as well (Robinson - 29).
- choice of inappropriate cultures - attempts have been made to market 'bio-yoghurts' containing high counts of *Enterococcus faecium*, and this practice must be regarded as dubious in the extreme. In the first place, *E. faecium* carries a gene(s) for resistance to penicillin, and hence there is an obvious risk of transfer to other bacteria, including potential pathogens, in the intestine. While secondly, the species occurs at low levels in the colon compared with other genera, e.g. *Bifidobacterium* spp. and appears to have no established beneficial function. Fortunately, the product marketed in Europe failed because of its appalling sensory properties, but its appearance on the supermarket shelves does highlight the need for Regulatory Authorities to be vigilant.
- exaggerated health claims could lead to the same problems that were encountered with the marketing of Acidophilus Milk during the early part of the last century, for the dividing line between the provision of 'sensible information' and 'misleading information' is difficult to define.

Chapter 4. CONCENTRATED YOGHURT AND RELATED PRODUCTS

Concentrated yoghurt (labneh) is manufactured from natural, stirred yoghurt, and some traditional examples are 'labneh' (Middle East), 'tan or than' (Armenia), 'Skyr' (Iceland) and 'Shrikhand' (India). More recently, Europeans have become accustomed to buying so-called 'Greek-style yoghurt', which derives its name from the fact that the major suppliers are based in Athens. A further range of products, such as 'Ymer' (Denmark) is derived from milk incubated with mesophilic lactic acid bacteria, with or without the presence of lactose-fermenting yeasts (Tamime and Robinson - 19).

Traditionally, most of these products were made by concentrating normal yoghurt using cloth-bags or animal skins to give products of 220 - 230 g l^{-1} total solids and fat contents of ~ 100 g l^{-1} (Tamime and Robinson - 32). Once removed from the container and stirred, the labneh provided an ideal base for the incorporation of ingredients like chives or cucumber (Τζατζικι) or sweetners like honey (Robinson - 33). However, while the product remained an essential component of diets in the Middle East, the labour-intensive nature of the process and problems of hygiene acted as 'brakes' on any real expansion of the market. The use of nozzle separators to concentrate warm, natural yoghurt to the desired level of solids-not-fat was seen as one way of modernising the system, and cream was blended-in at a later stage to yield a product of around 240 g l^{-1} total solids with 100 g l^{-1} fat (Tamime and Robinson - 9). However, while nozzle separation is widely used for the production of quark and is a comparatively easy system to operate, labneh produced in this manner tends to have a slightly 'grainy' texture. Exactly why the product acquires this texture does not appear to fully understood, but reports of the phenomenon were widespread.

Eventually, membrane filtration provided the solution (Ozer, Robinson, Grandison and Bell - 34) but, as the quality of the 'new' product had to match labneh made with the traditional 'cloth bag' system, one of my students, Barbaros Ozer, and I decided that a number of alternative approaches needed to be evaluated. Pre-concentration of the milk by ultra-filtration or reverse osmosis was one obvious option, while the application of membrane filtration to yoghurt of pH 4.6 or thereabouts provided another route. The direct reconstitution of whole milk powder to 230 g l^{-1} total solids followed by fermentation was considered to be a technically straight-forward approach but, as with the other options, little was known about the likely rheological properties of the product.

4.1 Background to the Study

Yoghurt gels are particulate structures, mainly composed of caseins, and the structure of such gels has been widely studied. Similarly, the effects of processing conditions, such as heat treatment, type of starter culture or level of total solids, on the properties of both set and stirred yoghurts have been evaluated using scanning electron microscopy. Depending on the processing conditions, continuously connected protein strands produce a heterogeneous three-dimensional gel network which holds free water, and any factors which affect the properties of the gel network by changing the nature and number of protein interactions also affect the water-holding capacity of the gel. The gel structure is known to involve both covalent (thiol/disulphide interchange) and non-covalent bonds, and the physical characteristics of particulate gels are determined by strong-permanent bonds (covalent bonds) formed during

coagulation, and by subsequent rearrangements of the casein aggregates. Furthermore, the final gel structure is also dependent upon the number of weak-reversible interactions that arise between the aggregates prior to formation of the permanent bonds; therefore, the balance between strong and weak bonds controls the rheology.

Another factor affecting the physical characteristics of yoghurt-type gels is the distribution of protein-protein bonds over the gel network. Several studies have investigated the relationship between protein concentration, distribution of protein-protein bonds and rheology of the resulting gels. In the case of homogeneous cross-linked particulate gels, all particles contribute equally to the network moduli. However, in non-homogeneous gels like yoghurt, thick protein nodes that include more than one protein-protein junction point are evident, and their contribution to the elasticity of the gel increases as the number of stress-bearing strands decreases.

However, while there have been some significant studies of gels with low total solids, the gel characteristics of materials with high solids contents, e.g. concentrated yoghurt, was uncertain. Consequently, our programme included the preparation of samples of concentrated yoghurt by a number of different methods, namely:

Traditional concentrated yoghurt (control): produced by holding yoghurt (160 g l^{-1} total solids - pH 4.3) made from reconstituted whole milk powder in bags of double-layer cheese cloth at 4 °C for 18-20 hours;

Concentrated yoghurt (Ultra-filtration (UF) - after fermentation): fermented milk (as above) was concentrated by UF at 42 °C immediately after fermentation. The pH value of finished product was, after cooling, ˜ 4.0;

Concentrated yoghurt (UF - before fermentation): made by concentrating

freshly reconstituted milk ((160 g l^{-1} total solids) at 50 °C to approximately 230 g l^{-1} total solids prior to fermentation to a pH of 4.3. In this case, both set and stirred products were made, and the pH of the finished yoghurts was ~ 4.0;

Concentrated yoghurts (Reverse Osmosis (RO) - after and before fermentation): prepared as with the UF products, except that an RO system replaced the UF one; and

Concentrated yoghurt (direct reconstitution): made by reconstituting the required amount of whole milk powder in water at 40 °C to give a base milk of 230 g l^{-1} total solids, which was then fermented to pH 4.3 - again both set and stirred products were made;

and then a determination of any contrasts in rheology that could be revealed by dynamic rheometry (Ozer, Robinson, Grandison and Bell - 34), confocal laser scanning microscopy (Ozer, Stenning, Grandison and Robinson - 35) and/or scanning electron microscopy ((Ozer, Stenning, Grandison and Robinson - 36).

In addition, the gelation profiles of milks with either 160 or 230 g l^{-1} total solids were examined (Ozer, Robinson, Grandison and Bell - 37).

4.2 Chemical Composition of the Products

In general, the protein (range: 88 - 92 g l^{-1}) and fat (range: 82 - 92 g l^{-1}) contents increased in the traditional and UF-yoghurts in line with the concentration factor but, given that the level of total solids were standardised, the same fractions increased in the RO- and direct reconstitution yoghurts to a lesser extent (range: 64 - 68 g l^{-1} for protein and 61 - 66 g l^{-1} for fat). The lactose concentration was reduced from 62 g l^{-1} (original milk at 160 g l^{-1} total solids) to 42 g l^{-1} in the traditional

yoghurt - slightly higher in the yoghurts concentrated by UF, whereas in the RO and reconstituted products, the lactose content increased (range: 82 - 91 g l^{-1}); the ash contents of the yoghurts revealed a pattern similar to lactose. Overall, it seemed likely that these contrasts in chemical composition would be sufficiently large to be reflected in the physical properties of the products.

4.3 Gelation of the Milks

The pH value of the unconcentrated milk (160 g l^{-1} total solids) reached pH 4.3 within 240 minutes during fermentation at 42°C but, in the samples with higher total solids, the decline in pH was slightly slower due to the higher buffering capacity of the concentrated milks.

During the early stages of incubation (0 - 90 min), no viscoelasticity was evident in any of the samples but, as the pH decreased, an increase in the complex modulus (G*) - associated with a transition from the 'liquid' to the 'gel' state - was observed (Ozer, Robinson, Grandison and Bell - 37); this change was evident from the sudden drop in the loss tangent (tan δ) and a marked increase in the concentration of ionic calcium [Ca^{2+}]. The 'gel' onset point for all samples was in the critical range for acid gel formation, i.e. pH 4.9 - 5.2, and seemed to be independent of the level of total solids, i.e. 160 or 230 g l^{-1}. After the gels had formed, the loss tangent (tan δ) remained almost unchanged for all samples throughout the incubation period, suggesting the formation of essentially similar network structures.

In contrast with most of the samples, the complex modulus (G^*) of the UF-milk sample did not reach a steady 'plateau' within the experimental time-frame, but

continued to increase over the entire period of incubation. The loss tangent (tan δ) values of the UF-milk were, however, similar to those observed with the other samples, and this pattern suggests that the same interactive forces were involved in gel formation in all cases, but operating, for reasons that were not clear, at different rates.

The gel development rate was highest in the base milk (160 g l^{-1} total solids), followed by the reconstituted milk (230 g l^{-1} total solids), and the RO and UF milks in decreasing order, i.e. the lower the level of protein, the faster the rate of gel development (Ozer, Robinson, Grandison and Bell - 37). However, the similarity in the 'gel onset' points indicates that, once the critical pH has been achieved, gelation occurs irrespective of the level of protein present; only consolidation of the gel is dependent on the actual concentration of protein.

4.4 Physical Properties of the Products

In general, the casein particles in the concentrated yoghurts were linked at random and formed a matrix filled with the liquid phase (whey). As the level of total solids increased, so the casein particle chains become shorter, the pore dimensions diminished and the density of the matrix increased. In the traditional, UF-after and UF-before fermentation yoghurts, much denser structures were observed compared to samples with lower protein contents (RO-before, RO-after and direct reconstitution yoghurts). Consequently, there was a close relationship between protein content, gel strength and the dimensions of the voids in the network, and only with the RO-after fermentation yoghurt did this correlation breakdown; the latter samples appeared to

have only small whey-filled 'compartments' in the gel networks.

The precise reason for this appearance was not established, but it may be that the relatively high pressure (2 MPa) applied to the warm yoghurt caused a breakdown in the protein matrix and, as a result, the broken fragments appeared to be distributed evenly. This view is supported, to some extent, by the fact that the UF-after fermentation yoghurt has a much stronger gel structure, which implies that the lower pressure employed during the process results in an 'even distribution' of relatively less-disturbed protein networks as against an 'even distribution' of protein fragments.

4.5 Behaviour of the Starter Cultures

The growth of *Str.thermophilus* was similar in all samples, with the exponential growth phase ending at around 120 minutes of incubation at 42°C, irrespective of the total solids level; at this point, the pH values were between 5.2 - 5.6. In pre-concentrated samples of milk (230 g l^{-1} total solids), the numbers of viable colonies of *Str.thermophilus* were slightly higher than in the other milks (160 g l^{-1} total solids) at the end of the log phase. This more rapid development could be due to a relative increase in stimulatory factors, such as whey proteins, and the enhanced growth of the culture in the UF concentrated milk may have been responsible for the higher level of acetaldehyde detected in the end-product.

During the next period of incubation, a stationary phase of growth for *Str.thermophilus* was observed, while *Lb.delbrueckii* sub-sp. *bulgaricus* grew more rapidly. In the samples with lower total solids, the growth of *Lb.delbrueckii* sub-sp. *bulgaricus* continued until end of the incubation (240 minutes) but, in the pre-

concentrated samples, the growth rate declined after around 180 minutes (Ozer and Robinson - 38).

A relationship between *Str.thermophilus* and acidity was also evident in all samples, and regression coefficients between *Str.thermophilus* and pH varied from 0.829 to 0.935. In the milks with lower total solids, the counts of *Lb. delbrueckii* sub-sp. *bulgaricus* were not markedly affected by acidity (r^2 between 0.536 and 0.668), but the effect of acidity became more important in milks with 230 g l^{-1} total solids (r^2 between 0.791 and 0.845). This contrast could be due to the fact that the dividing cells of *Lb.delbrueckii* sub-sp. *bulgaricus* were exposed to high levels of acidity over the prolonged incubation period used with the concentrated milks; in yoghurts made with concentrated milks, the titratable acidities in the end-products were higher than in yoghurts concentrated after fermentation.

A sensory panel confirmed that there was clear distinction between the UF and RO samples concentrated after fermentation, and the performance of the UF-yoghurt *viz-a-viz* the traditional product was most encouraging. Obviously careful process control will be essential with any membrane system, and an analysis of some products on sale in Qatar only served to highlight this point (Al-Jedah and Robinson - 39). How one manufacturer ended-up selling a 'labneh' with over 300 g l^{-1} total solids and 170 g l^{-1} fat remains a mystery, because none of the processes that we examined could give rise to products that analysed in this manner.

4.6 Effluent Problems

The above study established that the production of concentrated yoghurt

(labneh) is, in terms of the quality of the end-product, best achieved by separation of the whey from natural yoghurt by the traditional cloth-bag method or by ultra-filtration but, in either case, the waste material is an effluent (whey) with a Biological Oxygen Demand that precludes careless disposal. In industrialised countries, on-site treatment plants are common within the dairy industry but, in countries like Jordan, there is considerable pressure on companies to consider methods of disposal that give an economic return. If the end-product of the disposal system is one that is normally imported, then the potential benefits are seen as even higher.

As typical labneh whey in Jordan contains ~ 25 g l^{-1} lactose, fermentation provides one obvious solution, and two options were assessed. The first route involved the production of a mixture of acetic and propionic acids (Haddadin, Al-Muhirat, Batayneh and Robinson - 40) and, as both acids can be classified as 'food-grade' when produced by the fermentation of whey, it was assumed that one (or both acids) could find ready outlets within the local food sector. The second option was a fermentation of the whey to acetone and butanol (Haddadin, Batayneh, Batarseh and Robinson - 41) for, although both compounds can be manufactured more economically by direct chemical pathways, both have to be imported into Jordan at the present time.

In both cases, the fermentations proved to be feasible, and the labneh whey appeared to contain no compounds that were inhibitory to the fermentations. However, whether full-scale production would be an economically viable proposition was not assessed.

4.7 Products derived from Labneh

As mentioned earlier, there is large and growing demand in Europe for concentrated yoghurt but, whereas Europeans regard the product as a 'novelty' item, in the Middle East, labneh has long been viewed as a central component of the diet. In addition, the seasonal availability of local milk makes it essential that these limited supplies of milk protein are not spoiled at the high ambient temperatures that prevail, for even labneh is susceptible to damage by yeasts and moulds. Today, refrigeration is becoming widespread but, traditionally, two options for preservation found favour with peoples inhabiting the Gulf Region.

The first option was to take a 'bag' of labneh that has been draining overnight, and then place the bag under boards weighed down with heavy stones. As further whey was forced out of the coagulum, the total solids increased until it reached around 400 g l^{-1} and, at this point, the labneh could be removed from the bag for shaping into small 'golf balls'. These balls were then immersed in olive oil, and the labneh - or labneh anbaris as it was called at this stage - remained stable for months on end; 18 months is the shelf-life printed on the retail jars of labneh anbaris that are now available in supermarkets.

Our studies confirmed that this remarkable chemical and microbiological stability stems from the fact that the acidity of the product suppresses bacterial growth, while the comparatively anaerobic conditions will not allow yeasts, moulds or oxidative defects to develop (Keceli, Robinson and Gordon - 42).

Now-a-days, labneh anabaris is the most popular option for the long-term storage of labneh but, hundreds of years ago, the peoples in Iraq developed an even

more stable material, kishk. The first stage in kishk-making involved boiling whole wheat grains in water until the starch gelatinised, and then drying the grains in the sun. After coarse grinding, the flour was mixed with yoghurt, or more usually labneh, to form a thick dough. 'Lumps' of this dough were then hand-moulded into 'balls' of around 200 g, which were then pressed onto a metal tray and sun-dried. As these labneh-cereal 'balls' have an A_w of < 0.5, are non-hygroscopic and extremely tough physically, they can be stored in an open jar for two/three years without any detectable change in chemical composition or sign of microbial activity.

On reconstitution in water and heating, the porridge-like gruel makes an excellent foodstuff, in that it contains protein, available carbohydrates and dietary fibre, and it seems curious that societies outside of Iraq do not appear to have developed a high-nutrient product similar to kishk; the more so, perhaps, as porridge *per se* has been consumed as a breakfast cereal for many years. Similarly in Mexico, many village children are weaned on a gruel of ground maize, and yet the nutritional value of a maize-based kishk would be much better (Robinson and Cadena - 43). In addition, our studies revealed that a gruel derived from a fermented milk-cereal base could be just as acceptable to children in Mexico as the maize-based food (Robinson and Cadena - 44).

Overall, our attempts to improve the general perception of the basic product have been greeted with little enthusiasm (Robinson - 45). Even in the Arab world, reactions were muted, even though taste panels convened in the Lebanon rated the whole wheatmeal kishk as having an 'attractive flavour' (Toufeili, Melki, Shadarevian and Robinson - 46).

It may be, of course, that there is something unfamiliar about the taste of acidic gruels, but it is curious that kishk enjoys such a limited distribution.

Chapter 5. STUDIES OF TRADITIONAL CHEESES

Although crude forms of cheese were probably derived almost as soon as Man domesticated animals, the earliest records of cheesemaking are probably those from Ancient Egypt. Earthernware pots of the type still in use today to separate the milk for making the soft Egyptian cheese, Mish, were found in the tomb of King Horaha dating from around 3,200 BC, while both reed mats - employed to drain whey from the soft curd - and storage jars for finished cheese have been located in tombs built during the Roman occupation of the Nile basin.

By 332 BC, a more textured type of cheese, Domiati, had become established, and it is of interest that, like Mish, it is a cheese ideally suited to the conditions pertaining in the Middle East. Thus, the high ambient temperatures and poor standards of hygiene meant that products with high acidities and salt contents were the most amenable to preservation, and it is probable that many varieties popular with present-day consumers, such as Feta or Halloumi, are refinements of these early products. Variants of these primitive systems of production evolved with time, and most tribes with herds of cows, sheep or goats will have derived some form of cheese or fermented milk.

As these groups intermingled as the result of wars or casual migrations, so the art of cheesemaking spread. Climatic or other conditions would have imposed contraints in various ways and, as a result, distinctive local varieties emerged. The introduction of agents capable of rapid coagulation of the milk, such as extracts from plants like the Thistle (*Carduus* spp.), Fig (*Ficus carica*) or Thyme (*Thymus* spp.), would have offered one route towards some degree of standardisation of the making

process, but the role of the bacterial flora, i.e. mesophilic or thermophilic, could have been equally important.

Since these early days, numerous varieties of cheese have emerged to attain differing levels of status in the eyes of consumers but, even so, the essential nature of cheese has changed very little. It is still a product made by the coagulation of milk by enzymes and/or acid with some of the whey expressed from the finished curd, and this simple definition is broadly applicable to all varieties. Different conditions of processing and maturation can, of course, change dramatically the nature of the finished cheese, and these aspects have, with respect to Feta and Halloumi cheeses, been studied in detail during the course of this work. In particular, it was hoped that, by understanding more about the microbiology and biochemistry of the maturation of these cheeses, the products could be standardised to suit their ever-expanding commercial markets.

However, one fundamental aspect of cheesemaking concerns the quality of the raw material - milk, and the availability and essential characteristics of this basic ingredient provided my research group with some additional avenues for research.

5.1 The Milk for Cheesemaking

The milk from any mammal can, in theory, be turned into a cheese-like product but, for purely practical reasons, milk from domesticated animals has always dominated production. The primary source has tended to be determined by the vagaries of the local countryside, so that while bovine milk has been more readily available in lowland areas, mountain tribes have relied on sheep or goats as the sources of raw material. In the semi-arid climate of Cyprus, for example, Halloumi

cheese made from ovine or caprine milks is the only cheese of real local importance (Robinson, Haddadin and Abdullah - 47; Papademas and Robinson - 48), and many Mediterranean cheeses are made from these same base materials. Subtle differences between the various milks, such as levels and composition of the milk fat, can affect the nature and quality of any cheese produced but, in many cases, much depends upon the process. Our low-fat and reduced-fat Halloumi cheeses were, for example, quite reasonable products compared with the traditional cheese, but it is likely that boiling the cheese in salted whey tended to mask potential deficiences (Papademas, Norman and Robinson - 49).

The two major forms of protein that occur in milk, namely the caseins and the whey proteins, are the essential components of many cheeses but, in general, the caseins are the more important fraction. Thus, not only do the caseins represent around 70 - 80% of the total protein present in milk, but they are the proteins which form the matrix of the curd and, ultimately, the cheese itself; only if the cheese is made from milk that has been subject to ultra-filtration or the cheese is made from recombined milk (Ali and Robinson - 50) is the whey protein fraction of real importance.

During cheesemaking, the lipid content of the milk is largely retained in the curd along with the caseins so that, in a typical hard-pressed cheese, 45% of the total solids content may be fat. What is crucial, however, is the ratio between the fat and the protein. Obviously this point has been well-established for cheeses like Cheddar, but one of my students working in Jordan showed that it was equally relevant for the semi-soft cheese, Nabulsi (Haddadin, Shahin and Robinson - 51).

The remaining solids in a cheese will be low levels of lactose and mineral

salts, and the former is essential both for the further production of lactic acid by the starter bacteria and to support the growth of the non-starter lactic acid bacteria that contribute to maturation. In Nabulsi cheese, for example, we found that the natural microflora varied with the source of milk, and that the presence of certain species appeared to play a significant role in flavour development (Yamani, Al-Nabulsi, Haddadin and Robinson - 52).

5.2 The Role of Cultures in Cheesemaking

As with fermented milks, bulk starter cultures may be grown on-site or purchased for direct addition to the process milk but, given the potential problems with bacteriophage or poorly-balanced multi-strain cultures (Robinson and Ali - 53), the use of direct-to-vat cultures is increasing in popularity. The actual cultures are selected for their ability to produce lactic acid from lactose, and/or their ability to produce distinctive metabolites that contribute to the flavour profile of a given cheese. In the latter context, the correct choice is especially important for cheeses that undergo a period of maturation, in that: (a) the metabolic activities of the bacteria during the early stages of maturation create the reducing conditions necessary for the numerous biochemical changes associated with flavour development to occur; and (b) the release of various enzymes from dead cells again contributes to the complex changes that turn a raw curd into the finished cheese.

However, while both mesophilic and thermophilic genera of bacteria are widely used in cheesemaking, a number of traditional cheeses like Halloumi (Papademas and Robinson - 48) and Nabulsi (Yamani, Al-Nabulsi, Haddadin and Robinson - 52) are made from raw milk with no additional cultures being needed.

This system has served the local cheesemakers well for many years but, as levels of production of these traditional cheeses increases and safety concerns become more pressing, so the use of raw milk may have to be phased out. Switching to a procedure employing pasteurised milk and a commercial starter culture is the obvious option, but imitating the sensory properties of the traditional cheese is not easy. In some cases, the use of lactic acid bacteria isolated from local raw milk could allow producers to retain the familiar flavour of their products, and certainly this approach was found to be successful during limited trials with a fresh cheese from Jordan, Nabulsi (Haddadin, Shahin and Robinson - 51, 54).

If the cheese is one that is matured for several months then, even if pasteurised milk and starter cultures are employed, organisms of non-starter origin, such as lactobacilli from the environment, could prove to be central to flavour development. The precise relevance of non-starter lactic acid bacteria in the release of flavour compounds is difficult to determine in a natural biological material like cheese, but the high counts of lactobacilli found in many cheeses (Papademas and Robinson - 55) suggest that the genus must be making a very real contribution. This conjecture became relevant in this present study when it was established that Halloumi cheese made from ovine milk had a new species of *Lactobacillus* present, *Lactobacillus cypricasei* (Lawson *et al.*, 56). This species was never isolated from any samples of Halloumi cheese made from bovine milk, and it is tempting to speculate that the activity of *Lb. cypricasei* is one of reasons why Halloumi cheese made from ovine milk tastes different from its bovine counterpart (Papademas and Robinson - 57).

In cheeses like Feta and Halloumi that are stored in brine, yeasts could be as important as non-starter lactic acid bacteria in the development of flavour and texture.

Thus, the yeasts in brines surrounding Feta cheese were shown in one of our recent studies to be important sources of both lipolytic and proteolytic enzymes, and it would be surprising if these enzymes did not enhance the pungent flavour of the traditional Greek cheese (Bintsis, Litipoulou-Tzanetaki and Robinson - 58).

The practical significance of this observation is that it supports the tradition of Feta-makers in rural Greece of inoculating 'new' brine with a bucket-full of 'old' brine. Whatever the merits of this traditional system, it does raise fears over the safety of products destined for export, for our survey of the brines in cans of Feta cheese sold in the United Arab Emirates showed that *Listeria* spp. can survive during storage (Gohil, Ahmed, Davies and Robinson - 59). However, if cultures of yeasts isolated from traditional brines could be added to pasteurised brine, it is possible that a 'normal' process of maturation would be observed, and in a medium free from pathogens. Our work suggests that this approach could soon become a reality, but species and/or strain selection will be important, as will the rate of inoculation. *Yarrowia lipolytica*, for example, can be isolated from Feta brines quite frequently, but the strains that we recovered were so metabolically active that brines inoculated with 1.0×10^3 cells / ml gave cheeses that were totally inedible after maturation for 60 days. It is pleasing to record though that the same cell count of *Debaryomyces hansenii* added to a sterile brine produced an excellent cheese, and a field trial in Greece will commence shortly.

Alternatively, it is technically feasible to eliminate pathogens like *Listeria* spp. from cheese brine using furocoumarins and long-wave ultra-violet light (UVA) as the active agents (Bintsis, Litipoulou-Tzanetaki and Robinson - 60). Furthermore, populations of yeasts are less affected by the treatment, so that it becomes possible to

render a brine safe without destroying the microflora associated with the desired maturation (Bintsis, Litipoulou-Tzanetaki and Robinson - 61). Sadly, the current cost of furocoumarins and their ability to induce allergic reactions in humans means that the system lacks commercial potential. Nevertheless, a new generation of safe, synthetic furocoumarins is being developed for various medical uses, and hence the furocoumarin/UVA technique could still find an application within the dairy industry.

5.3 Aspects of the Cheesemaking Process

In the major dairy countries, cooled raw milk is transported to cheese factories in insulated tankers but, in less-developed countries, the initial handling of the milk can have a disastrous impact on its microbiological quality. In turn, these high bacterial counts are transmitted to the cheese, and this situation was well illustrated by a study of Bafut cheese from the Cameroon (Kameni, Mbanya and Robinson - 62). In this case, the milk was allegedly pasteurised and the equipment sanitised, and yet the samples of cheese analysed for pathogens failed to meet any normal specification. The logical conclusion was that the original milk was so heavily contaminated that neither the natural protective systems (Haddadin, Ibrahim and Robinson - 63) nor normal pasteurisation was able to cope with the bacterial load.

Where liquid milk is not available or is in short supply, recombined or filled milks can be considered as raw materials. In the Cameroon, for example, we successfully produced samples of the Bafut cheese from locally-available refined palm oil and imported skimmed milk powder (Kameni, Mbanya and Robinson - 64), and initial consumer reactions were promising. Unfortunately, a local civil war precluded further investigation.

In the Sudan, we employed an alternative and totally novel approach. In this case, skimmed milk powder, sodium caseinate and butteroil were recombined to give the levels of fat and total solids desired in the final cheese, and the cheesemaking procedure was modified accordingly (Ali and Robinson - 50, 65). Some physical differences between the experimental cheeses and the traditional local cheese, Gibna Baida, were obvious under the scanning electron microscope, but the sensory properties of the two cheeses were indistinguishable (Robinson and Ali - 66). Sadly, yet another civil war destroyed a factory being built outside Khartoum to manufacture commercial quantities of this recombined cheese, but at least the technology has been exploited by the New Zealand Dairy Board to encourage the production of a Feta-type cheese in the United Arab Emirates.

This success with the novel cheesemaking process encouraged us to explore the possibility of making a hard-pressed cheese based on soya milk, for in the home country of one of my students, Thailand, the distinctive taste of the soya bean is widely appreciated. A yoghurt culture was used to acidify a mix produced from reconstituted, spray-dried soya milk, and the activity of the culture was found to be excellent (Chumchuere and Robinson - 67). To our dismay, the texture of the resultant cheese was like 'putty', and the taste too 'beany' even for a taste panel of students from the Far East. However, when cubes of the cheese (2.5 cm^3) were fried in vegetable oil, the product was rated as 'excellent' (Chumchuere, MacDougall and Robinson - 68). Whether any entrepreneur in Thailand will try to scale-up the process remains to be seen, but it was one of our cheesemaking ventures that had a most unexpected ending.

5.4 Safety and Quality

Nowadays, the milk is normally pasteurised at 72°C for 15 seconds prior to cooling to 30°C and transfer to the cheese vat. Some small-scale producers, or those that insist on traditional technology, may still use raw milk, but scares over safety have made it illegal, or at least very difficult, for manufacturers in many countries to use raw milk for cheese making. In any event, there is little concrete evidence that the majority of consumers can tell whether a sample of cheese is made from raw or pasteurised milk (Nicol and Robinson - 69).

Once manufactured, the nature and duration of the maturation process varies with the type of cheese, for while fresh, unripened cheeses may be sold a few days after manufacture, cheeses like Feta are held for at least 60 days for the full flavour to develop (Bintsis, Litipoulou-Tzanetaki and Robinson - 58). The essential transformations involve changes in flavour, modifications to the structure of the cheese and a range of biochemical interactions associated with the extensive battery of compounds derived from the milk and/or the activity of various micro-organisms.

The mature cheese is, therefore, an extremely complex material, and hence judging the exact point at which a cheese has reached its optimum character is no easy matter. In general, the progress of maturation has to be judged subjectively by a skilled grader but, if necessary, objective schemes can be derived for use by less experienced tasters (Robinson - 70). In the final analysis, it is, of course, the consumer who decides on the acceptability or otherwise of a given product, and present evidence suggests that the brined cheeses investigated in this study will find an ever-expanding market.

Chapter 6 CONCLUSION

One of the problems that bedevils some fields of applied science in the modern world is that, in spite of their exciting potential, the work appears to lack glamour. Everyone admits that the tropical rain forests of South America may contain plants with medicinal properties that could be exploited for the benefit of patients across the globe, but few research organisations will divert money for the necessary searches. The field of food fermentations faces just the same lack of support, and yet many of these traditional processes have been central to the survival of rural communities over many years and there could be much to learn from an understanding of their chemistry and microbiology.

One example of this hidden potential emerged through a brief study that we made of the fermented milk from Kenya, *Iria ri Matii* (71). In essence, this product is made by pouring whole milk (often heated and cooled) into a hollowed-out fruit (gourd) from the plant, *Lagenaria leucantha*, and leaving it to ferment overnight at ambient temperature. Next morning, the slightly viscous and mildly acidic milk is poured out of the gourd and drunk by the children in the family.

Superficially, the fermentation seems simple enough, but it is the preparation of the gourd that is unique. Thus, after removing all the seeds and pith from the fruit, the inside wall of the gourd is rubbed with glowing pieces of wood from the mutero tree (*Olea africana*). This latter process may be repeated two or three times until the inner surface is smooth and then, after shaking out any excess ash, the gourd is filled with raw milk and left hanging at ambient temperature. Often this initial fermentation

gives a poor quality product that has to be discarded but, after several batches have been fermented and the gourd has been re-treated with glowing splints from the mutero tree, the fermentation stabilises. At this point, it can be assumed that a desirable microflora has built-up on the inner surface of the gourd, and that the organisms concerned are able to dominate any subsequent fermentations.

What remains curious, however, is the action of scouring with glowing splints. The obvious answer is that the heat is 'sterilising' the inner surface of the gourd, so that contaminant yeasts or bacteria do not prevent a desirable lactic acid microflora from developing. There may, of course, be some truth in this idea, but anecdotal evidence established that only wood from the mutero tree had the desired effect. 'Unscoured' gourds never gave rise to an acceptable product, and gourds cleaned with glowing pieces of wood from other trees or shrubs did not allow the establishment of a lactic microflora capable of producing *Iria ri Matii*.

In a global context, this fermentation from a remote corner of Africa may seem of little interest, and yet there must be a reason why only one type of wood will have this selective effect. If the active agent is chemical, could it have applications in another food context ?

To overlook such questions is all too easy, and it must be admitted that the studies that comprise this thesis have tended to concentrate on issues of more immediate relevance or, perhaps, were preceived as having a 'higher profile'. The simple method devised by one of my students for detecting antibiotics in milk supplies in Jordan provided a immediate solution to a very real problem, for importing ready-made test kits from Europe was never popular with dairies collecting ex-farm milk

supplies in small volumes.

Similarly, our studies on the effect of starter cultures on the quality of stirred yoghurt or the evaluation of media suitable for the enumeration of the bacterial floras in 'bio-yoghurts' were responses to perceived industrial needs and, in the latter case, a demand from consumer groups that the public should be protected from 'bogus' products that contained few viable cells of the desired therapeutic organisms. Publication in the popular consumer magazine, *Which*, of the counts for lactobacilli and bifidobacteria in named brands provided a useful reminder to manufacturers that 'therapeutic minimum' standards had to be maintained.

The study of the concentrated yoghurt (labneh) was again motivated by an industrial situation, in that a method for large-scale production was being sought as a matter of urgency. Initially, it was important to establish which of the techniques available to remove whey from normal yoghurt (~ 160 g l^{-1} total solids), i.e. ultra-filtration or reverse osmosis, would give a product that was almost 'identical' to traditional labneh (~ 230 g l^{-1} total solids) and, furthermore, to be able to demonstrate our conclusions objectively. In the end, the data generated by three different techniques of physical examination - dynamic rheology, scanning electron microscopy and confocal laser microscopy - confirmed that the structures of traditional labneh and a sample made by ultra-filtration were similar in all essential respects; a conclusion that was readily supported by sensory analysis.

Perhaps it is inevitable that anyone working with fermented foods wonders whether his/her work can be directed towards alleviating hunger or malnutrition in one of the less-developed countries, and our novel process for making a brined cheese to

match Gibna Baida from Sudan appeared to fall into this category. Thus, the process used ingredients available from Relief Agencies, was simple to operate and left no effluent, and hence it seemed entirely appropriate for Africa - especially as the sensory properties of the end-product were 'identical' to those of the native product. Perhaps one day the 'good intention' behind this project will be realised !

A more immediate 'intention' though is the desire of the Greek and Cypriot Governments to prove that their 'national' cheeses - Feta and Halloumi, respectively - are unique, and one of my students did discover some interesting properties of Halloumi. Plant terpenes from shrubs growing in the hills of Cyprus were found only in traditional ovine Halloumi cheese, as was a new species of the genus *Lactobacillus*, but whether such fine details will convince an International Court that Halloumi can only be made in Cyprus remains to be seen. It may be relevant, however, that Courts in the USA have already ruled that Halloumi cheese on sale in America must have been made in Cyprus, so perhaps the chemical and microbiological results published by my research group will serve to underline the fact that traditional Halloumi cheese really is unique.

While it has been gratifying to have the results of our research published in peer-reviewed Journals, equally pleasing is the fact that students from the Middle East, Greece and elsewhere have been associated with many of the studies described earlier. As these same students have then moved on to successful, science-based careers, the studies that they have completed in my laboratory gain an additional dimension and, as the author citations on the 'Submitted Publications' will confirm, many of the studies contain this 'hidden element'.

This 'teaching or training' component of the work served also to highlight deficiencies in the basic literature of dairy science, and books like *Yoghurt-science and technology* (Tamime and Robinson - 3) and *Dairy Microbiology* (not cited) were compiled to fill a perceived need. What was unexpected, however, was that their impact would spread beyond the English-speaking nations. In the event, *Yoghurt-science and technology* has been translated into Spanish and Farsi, while *Dairy Microbiology* has editions in Spanish and Arabic; even the rather specialist text - *Feta and Related Cheeses* (not cited) has a version in Farsi. Obviously research is first and foremost about 'originality', but when associated publications like review articles and books encourage students across the world to think more seriously about the cheeses and fermented milks that they may well consume everyday, the benefits of the research stretch well beyond the commercial application(s) of laboratory-generated data.

LIST OF SUBMITTED PUBLICATIONS

PREFACE

This Volume contains a series of original research papers and invited review articles that summarise the results of laboratory investigations of various fermented dairy products. In many cases, the papers - identified with the superscript * - were written with supervised research students as the co-authors but, in other cases, the student may have been supervised jointly for at least one component of the project - papers identified with the superscript +. The citation of three or more authors, i.e. one/two authors in addition to the student and co-supervisor, implies that a colleague(s) has provided specific laboratory facilities, e.g. a scanning electron microscope, not available within the Dairy Microbiology Unit. No superscript implies an equal partnership between the authors.

The title pages of some relevant books have been included in this Volume, but chapters contributed to books have been cited in the List of Total Publications only, as have Conference Proceedings and references to edited books; any of these latter publications can be made available on request.

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SUBMITTED PUBLICATIONS REPRODUCED IN FULL

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Size distribution of casein micelles in camels' milk

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The particle size distribution of bovine milk has been the subject of recent reports, and different techniques have been used to determine the range of micellar sizes. These techniques have included light scattering and centrifugal fractionation (Lin *et al.* 1971); electron microscopy (Schmidt *et al.* 1973) and chromatography and photon correlation spectroscopy (Griffin & Anderson, 1983). The size of the micelles ranges between 20 and 600 nm with a maximum frequency of around 60–80 nm as reported by Holt *et al.* (1978) and Kimber *et al.* (1978); submicellar particles were observed by Schmidt *et al.* (1973).

Despite its economic potential, the camel and its milk have received little attention. Moreover, the camel is well adapted to arid environments and can produce milk when its water intake is drastically restricted (Yagil & Etzion, 1980). Elamin (1980) gave an account of the dromedary camel of the Sudan and its milk constituents: the percentages of water, protein and fat were 86–90, 3·6–4·7, 4–5·5 respectively. Ahmed *et al.* (1979) studied some minor constituents of camels' milk: the amounts of Ca and P (mg/100 ml) detected were 137–312 and 39–152 respectively.

The casein micelle size of camels' milk is an important parameter of the structure of the milk. This was studied using transmission electron microscopy to observe the relative particle size distribution. Some observations on the structure of casein micelles, together with a comparison of their average diameter, are also given.

MATERIALS AND METHODS

Collection of samples

Six samples of milk were collected from six individual Bedouin camels (*Camelus dromedarius*), reared by nomads, from the Sudan. The samples were collected from two different semi-desert sites: (*a*) 50 miles N. of Wad Medani (capital of the Gezira Region), samples no. 1, 2 and 3 and (*b*) 10 miles S.W. of Wad Medani, samples no. 4, 5 and 6. This choice of sites enabled all the samples to be taken from camels within the first 1–2 months of onset of lactation.

To let down the milk, the baby camel (calf) was introduced to suckle its mother for about 10–15 min. Then after discarding the first 2–3 strips, about 2–3 ml milk were collected directly from each camel into test tubes containing an equal volume of 2·5 % glutaraldehyde in 0·2 M-sodium cacodylate-HCl buffer (pH 7·2), and then fixed for 1 h. Samples were mixed with an equal volume of 2·5 % molten agar (60–70 °C), and the material poured on to glass slides and allowed to set. The gel was then cut into 1 mm^3 pieces and kept refrigerated (below 5 °C) in 0·2 M-sodium cacodylate – HCl buffer (pH 7·2) ready for further processing, within 3 weeks, at Reading University, UK. Both immediate processing and alternative preparative techniques on fresh milk were impossible in these geographical sites.

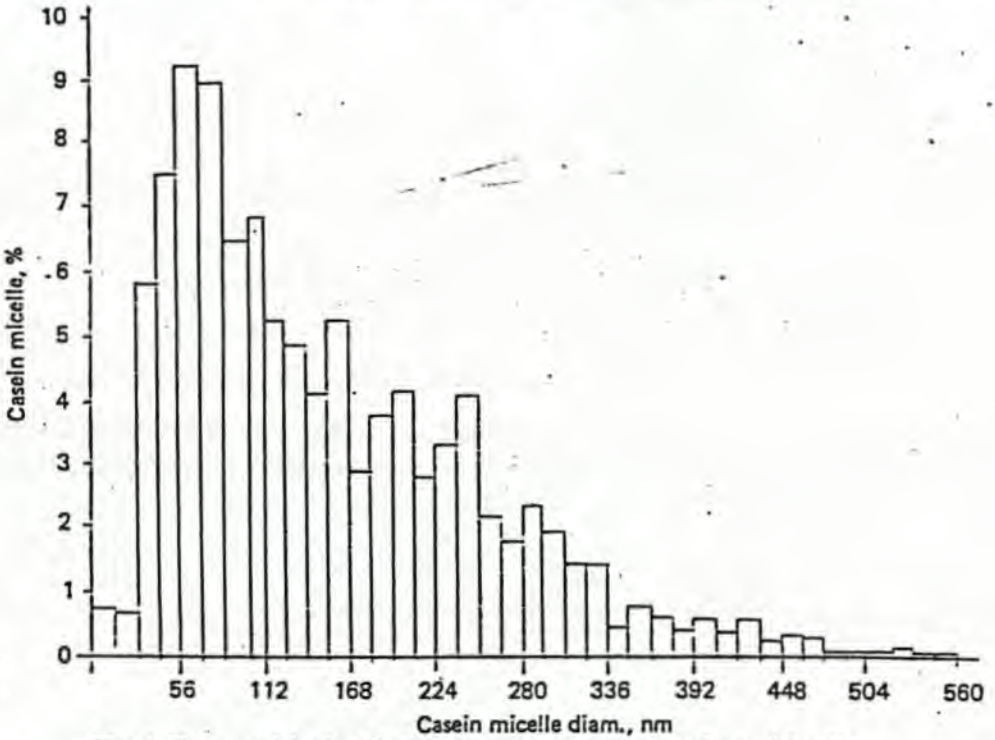

Fig. 1. True particle size distribution of casein micelles of camels' milk.

Table 1. *Apparent and true* number average diameter of casein micelles in milks from different camels*

Sample no.	†Apparent mean, nm	‡True mean, nm
1	163·9	167
2	165·4	160
3	163·9	160
4	163·8	162·9
5	166·2	159
6	157·5	149·8
Average	164·28	159·78

* Standard error = ±2·12
† Observed mean counted by the image analyser.
‡ Apparent mean after correction with the computer program.

Electron microscopy

The fixed material was rinsed quickly in a fresh buffer before post-fixation treatment in 1% buffered osmium tetroxide (pH 7·2) for 1 h. After rinsing in six changes of distilled water, samples were stained in 1% uranyl acetate for 30 min, dehydrated in a series of acetone–water mixtures (50, 70, 90%), and twice in pure acetone. They were then treated in propylene oxide and propylene oxide/Araldite mixture before embedding in Araldite.

Thin sections (about 80–100 nm) were cut with glass knives on a Reichert Ultramicrotome (Ultracut, Reichert-Jung, Slough, Berks, UK) and mounted on fine naked mesh grids. The grids were then examined in a transmission electron microscope (100 B, JEOL. Tokyo, Japan) operating at an accelerating voltage of 60 kV. Micrographs were taken randomly from fields with discrete casein micelles.

Fig. 2. Electron micrographs of casein micelles of camels' milk. (*a*) ×56000; (*b*) ×135000.

Two separate blocks of embedded material were sectioned in order to obtain more representative fields for examination.

Particle size determination

Transmission electron micrographs were prepared with a final magnification of ×36000, and the frequency distribution of the micellar sizes was determined with the aid of a semi-automatic image analyser (Model MOP 3, Reichert-Jung). The diameters of discrete micelles from each sample were measured separately on at least nine micrographs, and the results printed out into 40 classes, with a class limit of 14 nm (14–560 nm), *i.e.* particles of < 14 nm could be recognized but not sized. To calculate the true particle sizes, the apparent diameter classes of each sample were corrected by use of the computer program described by Goldsmith (1967).

RESULTS AND DISCUSSION

The relative frequency distribution of the diameter of casein micelles in camels' milk (total particles counted = 2448) is shown in Fig. 1. The observed pattern suggests that the majority of micelles have comparatively small diam. of 28–240 nm. The maximum frequency is observed at 56–70 nm, similar to that reported for bovine milk of 60–80 nm (Kimber *et al.* 1978; Holt *et al.* 1978). The range of size of casein micelles in the camels' milks was from the lower limit of measurement (14 nm) to 560 nm.

The number average diameter of casein micelles of the six camels' milks was 159·78 (Table 1). The apparent mean refers to that counted by the image analyser and the true one refers to the apparent mean after correction by the computer program. There are limitations on any preparative technique, such as plastic embedding of casein micelles, so that computed number average size is a function of the measuring technique; Brooker (1979) attributed differences observed between results of different workers of size range and distribution of bovine casein micelles to such differences in techniques. For this reason no comparison can be made here between sizes of bovine and camels' milk micelles. There was no significant difference between the observed and the true means of diameter average of casein micelles of the different camels' milks. Few mathematical procedures have been developed for correction of the apparent particle size of globular bodies such as that of the casein micelle (Goldsmith, 1967; Rose, 1980).

Typical electron micrographs of camels' milks are shown in Fig. 2(*a*,*b*). The ultrastructural appearance of the casein micelle was similar to that seen in other species (Knoop *et al.* 1973; Tan *et al.* 1971). Further analysis of the casein fractions, and of their position in the micelle network, is needed in order to explain the nature of the camel milk proteins and their chemical behaviour.

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Goat's milk utilisation for fermented milk products

R K Robinson and Ioanna Vlahopoulou investigate the reasons underlying differences in processing behaviour between goats and cows milk, based on comparisons of chemical composition

In many areas of Europe, the goat has always occupied a central position in the rural economy and many traditional products, such as the Saint Maure and Pelardon cheeses of France, provide ample evidence of this long association. Nevertheless, in relation to the overall development of dairy products, cow's milk has been the dominant interest and it is only recently that the potential of goat's milk as a raw material has been seriously assessed.

Initially, this expansion of interest reflected the fact that allergic reactions triggered-off, in susceptible patients, by the proteins in bovine milk were not observed after consumption of caprine milk.

This somewhat marginal interest in the milk has been further encouraged by the introduction of quotas on cow's milk within the EEC. The end-result is that goat's milk is now widely available within a rather specialised market sector, but a sector that still has relatively few products for immediate sale. Liquid milk and cheese were the obvious options to fill the void, but it was not long before yogurt was targetted as a feasible alternative.

The properties of yogurt manufactured from caprine milk are entirely different from those of the bovine equivalent. The reasons for this contrast have yet to be fully resolved. The fact that yogurts based on goat's milk tend to fare badly in comparison with the usual commercial product has added some urgency to the situation.

The aim of this review is to consider:
(a) some of the possible reasons for the differences in behaviour that are observed during the production of yogurt; and
(b) how many alleged deficiences in the characteristics of caprine products might be corrected.

Composition of the milks

The natural starting point for any comparison is the gross chemical composition and a number of recent papers have

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Interest in goat's milk and related dairy products is growing, with potential for introducing new products into the market.

Table 1. Some analyses of goat's milk that have been reported in the literature, together with typical analyses for both cow's milk and goat's milk; all figures as percentages. References in brackets.

Country	Breed	Total solids	Fat	Protein	Lactose	Ash
Australia (5)	Saanen	11.7	3.4	3.2	—	—
Egypt (6)	Baladi	13.9-15.8	5.2-6.8	—	—	—
Greece (7)	—	11.3-14.8	3.0-5.6	3.1-3.8	4.3-4.7	0.75
Libya (8)	—	13.0	4.0	3.8	4.4	0.9
Spain (9)	—	13.2-15.9	4.4-6.9	3.5-3.7	—	0.8
Typical Analyses						
Goat (10)	—	13.0	4.5	3.3	4.6	0.6
Cow	—	12.6	3.7	3.4	4.8	0.7

reported on the major chemical components of goat's milk. A summary of these results is shown in table 1; it is notable that the variations in respect of both fat and protein can be considerable. The causes of these differences are said to be breed, stage of lactation, geographical location/season and diet (1, 2, 3).

The influence of season and stage of lactation were considered to be of major importance; milk produced during the winter months had higher levels of fat and SNF (including protein) than milk taken during the summer (4). According to Mashaly *et al* (3), the values for fat and total solids increased during the early stages of lactation in milk drawn from Egyptian Baladi goats, but other figures (4) indicate a rather different picture. The reasons for these disparate findings are not obvious, but with so many factors involved, variability is only to be expected.

This conclusion may not be much consolation to the factory manager faced with the problem of deriving an end-product to a routine specification, but while herd sizes tend to be small and scattered across a range of geological sites, standardisation of the raw material will continue to be a problem. If bulk supplies become available, then it is possible that the typical analysis shown in table 1 could become the 'norm' and it is noticeable that the suggested figures do not differ greatly from those reported for cow's milk.

Obviously, seasonal and other variations may be important to the manufacturer of a dairy product, but the contrast in behaviour between cow's milk and goat's milk during fermentation processes suggests that differences, either quantitative or qualitative, between discrete components of the milks may be more critical than the overall figures.

Lipids

An important characteristic of the fat globules in goat's milk is that they are smaller in size than those in cow's milk (11, 12), although Singh *et al.* (13) found that pasteurisation at 61°C for 30 minutes increased the average globule size by about 12% as a result of coalescence. In addition, it has been established that the fat globules of caprine milk do not cluster when milk is cooled, apparently due to the lack of the agglutin which is found in bovine milk (14).

Cerbulis *et al* (15) studied the lipid distribution between the cream and skim-milk fractions that arise as a result of centrifugation of goat's milk and in addition, analysed the composition of the lipid fraction of whole milk. This composition and particularly the dominant position of the triglycerides, is not unlike that of cow's milk (16), but it was noted that the skim-milk fraction of caprine milk contained significantly more free lipids than did the bovine equivalent.

On storage, Patton *et al* (17) observed an increase in the number of free phospholipids, suggesting that the fat globule membrane may be susceptible to damage. If this conclusion is correct, then the release of short-chain fatty acids from the globule core could explain the build-up of 'goat flavour' during the storage of raw milk. It might imply also that milk from breeds secreting 'thick' fat globule membranes would be more mild in flavour, but this possible correlation does not appear to have been explored.

The free fatty acids found in milk are mainly the result of hydrolysis and their presence can result in desirable or undesirable flavours, depending on both the type present and their concentration. In caprine milk, the free fatty acids are the main source of the characteristic 'goaty flavour', and a concentration of 5.65 meq/litre (total free fatty acids) is enough to provide an unpleasant 'note' (18).

Stark (12) attributed the characteristic flavour of goat's milk to the relatively high levels of short and medium chain-length fatty acids. A number of lipases are responsible for the observed hydroloysis (19) and the majority are sensitive to heat-treatments; the pasteurisation of milk immediately after collection could, therefore, be instrumental in reducing the extent of hydrolysis, and hence the build-up of undesirable flavours.

Overall, it is clear that the chemical differences between caprine and bovine lipids are sufficiently pronounced as to explain the more obvious contrasts in flavour associated with their presence in a dairy product. Whether there are parallel differences in respect of the physical properties is not well established, but the impact of the two milks on the textural characteristics of a cheese, for example, suggests that a comparison might be of interest.

The physical properties of fermented milks, however, are mainly dependent on the behaviour of the milk proteins, and it is in relation to these components that the comparison between caprine milk and bovine milk may become most relevant.

Proteins

The proteins in goat's milk have been less extensively studied than those in cow's milk (20), but various workers have

Yogurts based on goat's milk have tended to fare badly in comparison with the typical commercial product.

Table 2. The range of reported figures for the major protein fractions of goats' milk, together with a typical figure for cow's milk; all figures as percentages in normal milk.

Proteins	Goat's milk	Cow's milk
Total casein	2.14-3.18	2.6
αs — casein	0.34-1.12	1.28
ß — casein	1.15-2.12	0.8
K — casein	0.42-0.59	0.37
Whey proteins	0.37-0.70	0.8
ß — lactoglobulin	0.18-0.28	0.3
α — lactalbumin	0.06-0.11	0.07
Serum albumin	0.01-0.11	0.03

After: Juarez & Ramos (9); Banks et al (16).

isolated and characterised the major components; a summary of their results is shown in table 2.

The structure of the bovine casein micelle has been the subject of serious investigation for a number of years, and according to a recent review (21), the four major components, αs_1–, αs_2–, ß- and κ-casein, form aggregates or submicelles that coalesce together with mineral material to form the micelles. Kappa-casein tends to be located on the surface of the micelle, whereas α_{s1}-casein is implicated in the internal structure. Ono and Creamer (22) used gel filtration chromatography to separate casein micelles and sub-micelles (aggregates) according to their size, and they found, as did Richardson *et al* (23), that the caprine micelles were smaller than bovine ones. The micelles contain little αs_1– casein (23) and the major component appears to be β-casein (22).

However, when the size distribution of caprine casein micelles was studied by electron microscopy (24), it was found that the micelles ranged in size from 100 to 400nm in diameter. The majority fell into two categories — 100-200nm (35%) and 200-300nm (42%) — and these figures provide an interesting comparison with the studies of Holt *et al* (25) on cow's milk. Thus, the latter workers found that while the casein micelles ranged in diameter from 20-600nm, the maximum frequency occurred around 60-80nm.

These observations would suggest, therefore, that the micelles in caprine milk are, on average, larger than those in bovine milk, a feature that could explain, at least in part, the contrasted behaviour of the two milks during fermentation processes.

Use in fermented milks

Although yogurt has been made with goat's milk for a number of years, the final product tends to be different from cow's milk yogurt in a number of respects that are important to the consumer. In particular, it has been commented that:

(a) the caprine produce often lacks the typical flavour of yogurt;

(b) the coagulum tends to be rather soft and often lacks the pleasing mouthfeel of cow's milk yogurt; and

(c) on the positive side, it has been found that retail products rarely show any signs of syneresis.

Additional features have been reviewed by Loewenstein *et al* (26), but it is noticeable that many of the reports are somewhat contradictory. Thus, Aggarwal (27) produced a sample of set yogurt from goat's milk containing 4.3% fat and 8.6% solids-not-fat, and recorded that the end-product was indistinguishable from the control made with cow's milk. Duitscheaver (28) and Loewenstein *et al* (29, 30) found that yogurts made from comparable milks were readily identified, and that the product from goat's milk was less viscous. The former investigator also noted that acid development was more rapid in goat's milk, a point that was later confirmed by Manjunath *et al* (31) and by the authors (unpublished data).

The inconclusive nature of some of these conclusions is no doubt a reflection of the extreme variability of the raw material and differences in performance between the starter cultures used. But despite these problems, a number of attempts have been made to improve the organoleptic characteristics of goat's milk yogurt.

The influence of homogenisation has been studied on the quality of yogurts made from milks concentrated by vacuum evaporation, reverse osmosis, ultra-filtration or the addition of skim-milk powder (goat's milk); it was concluded that homogenised, ultra-filtered milk gave rise to products with optimum flavour and viscosity. Fortification of goat's milk by ultra-filtration was confirmed as the most suitable approach for producing a satisfactory yogurt (32), and it was suggested that the increased threonine residues in UF milk might explain the marked improvement in aroma recorded in the end-product.

The possible impact of free fatty acids on the flavour of goat's milk yogurt has also been the subject of speculation and it has been shown by Boccignone *et al* (33) that caproic, caprilic and linoleic acids all increased during yogurt production. The implication of these changes in respect of flavour could be important, and hence the selection of the starter culture may be critical. More recently, Rysstad and Abrahamsen (34, 35) have shown that the stage of lactation can exert a major influence on starter activity, in that the production of acetaldehyde, for example, increased in late lactation milk to reach 8.9ppm; this level is still well below that associated with cow's milk yogurt.

Conclusion

Overall, it would seem reasonable to conclude that there are definite differences in the texture and consistency of yogurts made with the two milks, and that these differences are a reflection of the contrasted micellar structures. Thus, it could be argued that the larger micelles in caprine milk aggregate in the presence of acid to give a softer and less compact coagulum than that associated with products of bovine origin, as this conclusion would explain not only the apparently less attractive mouthfeel of goat's milk yogurt, but also the resistance of the gel to further shrinkage and syneresis during storage.

If this conclusion is correct then, improvements in the textural properties will have to centre on the employment of either ultra-filtration, appropriate stabilisers or specially chosen starter cultures.

The latter course would, for a number of reasons, be of most immediate interest to many small manufacturers and, certainly in cow's milk, the choice of starter culture can have a dramatic effect on the physical characteristics of the retail product (36).

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YOGHURT

SCIENCE AND TECHNOLOGY

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Contents

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The detection of inhibitory substances in ex-farm milk supplies

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ABSTRACT

It is vital to ensure that milk employed for making cheese or yoghurt is antibiotic-free. Test kits are available commercially for ß-lactams and sulphonamides, but less expensive and more readily available techniques may needed for use in the less developed areas of the world. A simple procedure employing a yoghurt culture has been devised to give a result in 2.5 hours and, as this timing is identical to many commercial tests, the new test could be used as part of the reception programme at a small dairy.

INTRODUCTION

Although lysozyme and lactoferrin can be present in mastitic milk at sufficiently high concentrations as to cause false positives in antibiotic assays (1,2), these natural antimicrobial systems are unlikely to affect the starter cultures employed for cheesemaking, while the heat-treatments applied during the manufacture of fermented milks will cause total inactivation.

Detergent and disinfectant residues can also affect culture activity adversely but, in general, the levels required to induce inhibition are so high that only extreme negligence leads to problems from this source.

However, antibiotics have been used in dairy management programmes for decades, and their application is essential for the control of mastitis and other bacterial infections (3,4). Such antibiotics can easily pass into milk and, unless contaminated milk is discarded, it

can prove a major problem for the receiving diary (5,6). In some countries, antibiotics are employed as feed additives to enhance milk production (7), and residues from this practice could find their way into the food chain.

Such uses of antibiotics remain controversial (8), so that the most likely route for antibiotic contamination of milk remains direct administration. The dose given to any specific animal will depend upon the type of antibiotic used, the severity of the infection, the frequency of administration and the judgement of the vet (9), so that levels of administration vary widely. This variation is one of the factors that affects the withholding time of the milk, a period defined as "the time from the last injection or infusion of antibiotic to the time of drawing of the last milk sample to test positive for the antibiotic"(10).

In general, around 20% of an antibiotic will be secreted in the first milking after administration, and a classic study in the USA showed that, if 100,000 International Units (IU) of penicillin were administered by intra-mammary infusion, the level of antibiotic in the first and second milkings was 8.15 IU/ml, and 0.042 IU/ml for the sixth milking (9). No pencillin was detected thereafter, and hence the general recommendation has become that milk should be discarded for three days following treatment.

What is important, however, is that other factors, such as the yield of milk from a given animal, the physical health of the animal and the carrier of the antibiotic (aqueous or oil), can all affect the persistence of a specific antibiotic residue (11). One survey, for example, showed that 21% of samples tested positive beyond the recommended holding period (12), a rate of failure that was deemed to be far too high. In part, the result was attributed to the fact that current analytical methods are much more sensitive than those employed in the past (11), but it served to highlight two issues: (i) is it undesirable for antibiotic residues to be present in milk ? and (ii) what methods are available to test raw milks, what is their sensitivity and are they suitable for use in industrialised and less developed countries alike ?

IMPACT OF ANTIBIOTIC RESIDUES ON HUMAN HEALTH AND DAIRY TECHNOLOGY

An International Unit is defined as 0.6 μg of the sodium salt of benzyl penicillin (13), and levels

as low as 0.002 IU / ml of penicillin in milk can cause partial inhibition of dairy starter cultures (14). The absence of antibiotics in milk used for cheese or yoghurt production is, therefore, essential, but an additional problem is the apparent interference of antibiotics in the phosphatase reactions (15). In practical terms, this possible invalidation of a test which is widely used to monitor the adequacy of pasteurisation could have serious implications for public health, irrespective of whether the milk is for direct retail sale or for the manufacture of a non-fermented dairy product.

If the food manufacturer is concerned about potential technical problems, of equal concern is the possible affect on human health. In some countries, milk containing antibiotic residues is rated as adulterated (9), and hence its sale would be regarded as illegal. The principle behind this stance is that many adults are hypersensitive to penicillin and its derivatives, and a dose as low as 0.003 IU / ml of milk can cause a variety of allergic reactions ranging from mild skin rashes to death (16). The influence of antibiotics on the normal microfloras of the human intestine is well documented also (17), as is the risk that any decline in the native populations in the gut may permit undesirable bacteria or yeasts to flourish and over-grow sectors of the intestinal wall. In addition, the ingestion of residues may increase the number of antibiotic-resistant bacteria in the intestine, and certain species from within these antibiotic-resistant microfloras may transfer the gene(s) for resistance to potentially infectious species. When this transfer happens, clinical treament with the same antibiotic will be ineffective, and the search for a cure can be both time-consuming and distressing for the patient (13).

It is this possible scenario that has lead the Federal Drug Agency (USA) to specify a 'zero tolerance' for antibiotics in milk and milk products. In practice, of course, this regulation has to be intrepreted as the lowest concentration of the antibiotic which is detectable by an approved technique (17). Bearing this latter point in mind and the data available concerning the potential risks to consumers (18), the WHO/FDA produced guidelines covering the maximum concentrations of antibiotic residues to be permitted in foods (19). These figures are summarised in Table 1.

Obviously these maximum concentrations are a reflection of the levels that the WHO/FDA feel can

Table 1. The suggested maximum concentrations of antibiotic residues in milk.

Antibiotic	Concentration (mg/litre)
Ampicillin	0.01
Bacitracin	1.20
Cephalosporins	0.01
Chloramphenicol	0.00
Chlorotetracycline	0.02
Cloxacillin	0.02
Erythromycin	0.04
Framycetin	0.15
Nafcillin	0.02
Neomycin	0.15
Novobiocin	0.15
Nystatin	1.10
Oleandomycin	0.15
Oxytetracycline	0.10
Penicillins	0.006
Polymixin B	5.00
Streptomycin	0.20
Sulphonamides[1]	0.10
Tetracycline	0.10

[1] Sulphonamides are often used on farms to control superficial infections and they will inhibit starter cultures for cheese or yoghurt.

be detected by monitoring on a routine basis, and this approach is crucial for success in eliminating residues from milk.

CONTROL OF RESIDUES IN MILK

The only practical method of controlling residue levels of antibiotics in milk is to institute a rigorous programme of testing. However, if such programmes are managed by a Central Authority, the system may involve no more than the testing of milk from a given herd once a month (20). Thus, milk that is satisfactory on the day of testing might fail a few days later as a result of antibiotic therapy being resumed. The daily testing of milk at a receiving dairy offers a better option, particularly if individual farmers make use of on-farm test kits as well (6, 21). However, the financial penalty that accompanies the withholding of milk for three days or more remains a constant encouragement to the practice of diluting contaminated milk with good quality material.

The FDA has attempted to improve the situation by requesting that drug companies recommend a minimum effective dosage for each antibiotic therapy, and place a clear warning on the preparation about withholding times. In addition, Schoech (22) published a detailed series of guidelines for farmers, but the financial loss associated with

withholding milk does place a burden on small farmers; although illegal, some farmers in the USA are believed to treat milk with ß-lactamases in order to avoid antibiotic failures (13, 23).

Given this obvious conflict between the necessity of the farmer to sell milk and the desire of cultured product manufacturers and the general public to avoid problems arising from exposure to antibiotics, the debate returns to the starting point - the need for a rigorous programme of testing. Exactly how such a programme should be carried out will depend upon the political infra-structure within any given country, but equally important is the question - are the techniques of detection reliable enough ? In particular, it is essential that any selected procedure:

(i) provides results that are accurate and repeatable for any given sample, and reproducible between different laboratories; there must be no room for disputes between parties about the validity of the test;

(ii) provides results that are available within a time-frame that allows for rejection of a suspect batch before production of a fermented product is lost, or a bulk silo of market milk is contaminated; and

(iii) is not so expensive as to limit its use within the country in question.

AVAILABLE TEST PROCEDURES

The factors to be considered when choosing a test method include: the type of antibiotics likely to be encountered, time limitations, degree of sensitivity, cost and, perhaps, any requirements laid down by Regulatory Authorities (24). In general, the majority of tests rely on the ability of a selected species of bacterium to produce acid or reduce a dye in antibiotic-free milk, or produce visible growth on an agar medium supporting discs of filter paper impregnated with the test milk. If antibiotics are present, then the rate of metabolism and/or the rate of cell division is reduced, and the effect can be readily detected by the absence of a change of colour or no visible colonies around the disc of filter paper. In many countries, users are allowed a choice of nominated test procedures, and amongst those available are:

Cylinder Plate Method

In this procedure, a basal medium is seeded with the test bacterium, eg. *Micrococcus lutea* (formerly *Sarcina lutea*) (25) and

poured into Petri dishes. After solification, short lengths of glass tubing (warmed to create a seal with the surface of the agar) are located around the plate. A different test sample of milk is then pipetted into the open end of each tube; antibiotic standards will be included on each plate. After incubation at 30°C for 18 h for *M: lutea*, the glass tubes are removed and the diameters of any zones of inhibition can be measured with calipers to the nearest 0.01 mm and compared with zone(s) of the antibiotic standards.

The sensitivity is quoted as being around 0.01 IU penciliin / ml of milk but, as the test is non-specific, confirmation with penicillinase is a necessary part of the procedure. A further increase in sensitivity was obtained by adding lysozyme from egg white to the medium to give larger zones of inhibition (26); the detection level was reduced to 0.0075 IU penicillin / ml of milk. However, although the reliability and sensitivity of the procedure is good, it demands a high level of technical expertise and the results are not available for around 24 h. Nevertheless, it remains a recognised Standard Method in the USA (27).

Disc Assays

Full details of this technique are provided in the Standard Methods from the USA (27), but essentially it involves:

- preparing plates seeded with around 10^6 spores of *Bacillus stearothermophilus* var. *calidolactis*
- touching discs (12.7 mm diameter) of filter paper onto the surface of a well mixed sample of test milk, and allowing a specific amount of milk to be absorbed by capillary action
- placing the test discs (up to five) and a control disc containing 0.05 IU penicillin in a regular circle 1 cm from the edge of the Petri dish
- incubating the inverted plates at 64°C for 2.5 h and measuring the zones of inhibition.

The rapidity of the test means that it can be employed to screen incoming milk to a cheese or yoghurt plant, and the sensitivity is around 0.008 IU penicillin G / ml of milk (29); again a penicillinase treatment is required to confirm the presence of a ß-lactam residue. An improvement in repeatability and reproducibility has been obtained by placing dry filter paper discs onto the surface of the agar and then pippetting 90 μl of test sample onto the disc.

A modification of the technique using *Bacillus megaterium* to detect sulphonamides has been devised (30). Although again demanding on laboratory expertise, the Disc Assay remains the preferred

choice of a number of authorities around the world.

Delvotest-P/SP

This test again employs the antibiotic sensitivity of *Bacillus stearothermophilus* var. *calidolactis*, but in a manner that is extremely 'user friendly'.

Small ampoules of basal medium containing bromocresol purple as an indicator and the bacterium are purchased ready-prepared, along with a bottle of nutrient tablets (31). To run a test, 0.1 ml of milk plus one nutrient tablet is added to an ampoule, and the complete mixture is incubated at 64°C for 2.5 h. A positive control (0.008 IU penicillin / ml) and a negative control (antibiotic-free skim milk) are set-up at the same time.

In the absence of any inhibitory substances, the bacterium secretes lactic acid which changes the medium from purple to yellow, while the retention of the purple colour indicates no metabolism by the organism; a green colour is regarded as a doubtful result that necessitates further examination. The sensitivity of the test to a range of antibiotics is shown in Table 2, and the test is regarded as especially appropriate for the residues of penicillin G.

The range of sensitivities to

Table 2. Lowest detection concentration of different antibiotics to Delvotest-P; all concentrations as μg/ml of milk except pencillin (IU/ml) (31).

Antibiotic	Sensitivity
Penicillin	0.004
Cloxacillin	0.025
Ampicillin	0.003
Streptomycin	8.000
Neomycin	6.000
Tetracycline	0.200
Erythromycin	1.750

other compounds, eg. chloramphenicol (5.0 - 8.0 μg/ml) and sulphonamides (50.0 - 100.0 μg/ml)(31) suggests that the strain of *B. stearothermophilus* var. *calidolactis* has been selected primarily to cope with antibiotics. This view is borne out by the fact that a new test, Delvotest-SP, has recently been introduced to monitor for sulphonamides alongside ß-lactams. In this test, the sensitivity of the test bacterium to sulphonamides is enhanced by the introduction of trimethoprim into the medium, so that the level of detection is lowered to 0.25 - 1.0 μg/ml, depending upon the compound.

The procedure is simple to carry-out, not too demanding

technically, and it has become the approved method of testing in many countries within Europe. However, confirmation of the nature of the inhibitory material may be needed; penicillinase to confirm for ß-lactams, and para-aminobenzoic acid to test for the presence of sulpha residues. The cost *per* test is less than £ 1 (Sterling), but even this routine outlay could deter small dairies in less developed countries.

A rather similar test - the Valio T101 Test - has been marketed in Finland but, in this case, *Streptococcus thermophilus* (T101) is the test organism. The sensitivity of this strain to streptomycin, neomycin and erythromycin is reported to be better than the figures shown in Table 2, but the level of detection of penicillin is the same (32).

Charm Test

This test is among those adopted by the AOAC (29), and the original test depended upon the affinity of ß-lactam antibiotics for specific sites on the cell walls of bacteria. In practice, penicillin labelled with ^{14}carbon is mixed into a sample of milk (5 ml) along with a suspension of *Bacillus stearothermophilus*, and the mixture incubated at 90°C for 3 m. The cells are then removed by centrifugation, dried and the level of radiation determined. If ß-lactams are present in the milk sample, then these compete with the ^{14}C antibiotic for sites on the bacterial walls, and hence the radioactivity in the precipitated cells is reduced compared with an antibiotic-free control.

Collaborative studies confirmed detection at 0.01 IU / ml of ß-lactams, but it is technique that could prove expensive with respect to laboratory equipment and technician expertise. The same is true for the more recent generation of Charm Tests that employ ^{3}H-labelled reagents to detect a range of different antibiotics (33). As might be anticipated, the sensitivity is excellent, and the sort of results

Table 3. Lowest detection concentration of different antibiotics in the Charm Test II; all concentrations as µg/ml of milk (34).

Antibiotic	Sensitivity
Penicillin	0.003
Streptomycin	0.010
Novobiocin	0.010
Tetracycline	0.600
Erythromycin	0.020
Sulphonamide	0.050
Chloramphenicol	0.080

shown in Table 3 can be obtained in around 15 m.

Alternative Systems

The use of antibiotic preparations containing dyes like Brilliant Blue F.C.F has been recommended in some countries (35, 36), but it must be established that the dye is secreted into the milk at the same rate as the antibiotic(s) in popular use. Obviously the presence of dye is a strong deterrent to any producer to include contaminated milk in a market load, but the general impression remains that the procedure is not reliable enough for routine purposes (37)

Enzyme Linked Immunosorbent Assays (ELISAS) for the detection of ß-lactam antibiotics have gained formal approval in some countries, and the Delvo-Xpress (Gist-brocades, Holland) and the Lactek (Idetekk, USA) systems, for example, are being promoted on account of the speed and sensitivity. Thus, 0.006 IU of penicillin G / ml of milk can be detected in 5 - 7 minutes using the Delvo-Xpress (31), and hence milk coming into a dairy can be rapidly screened before further processing.

Alternatively, this rapid derivation of results means that Regulatory Authorities could screen a large number of samples of market milk in order to protect consumers from antibiotic residues. As with many ELISA systems, the cost *per* test will be in the region of £ 1 - 2 (Sterling) but, in many situations, the speed and specificity of this approach justifies the expenditure.

CHOICE FOR THE USER

It is clear from this review of the procedures available to monitor milk supplies for antibiotics and other inhibitory substances that potential users have a range of options. Obviously cost may be a major consideration - investment in laboratory equipment has been ignored in the figures given above, as may routine access to the consumables necessary to complete a test.

Nevertheless, the consuming public deserves protection, and milk processors must be able to monitor supplies in order to avoid the financial losses that can follow from failure of a fermentation. It is probable that the Public Health Authorities in most major conurbations can afford to select the test(s) that is most appropriate for their needs but, for the dairy industry, the decision may not be so clear-cut. Dairies in rural areas, for

example, may have especial problems with regard to access to the latest technology, and it was with situations like this in mind that this project was instigated.

A SIMPLE TECHNIQUE FOR MONITORING MILK SUPPLIES FOR INHIBITORY SUBSTANCES

Most cheeses and fermented milks are manufactured using either:

(i) A mesophilic culture consisting of *Lactococcus lactis* sub-sp. *lactis* and *Lactococcus lactis* sub-sp. *cremoris*, along with, in some cases, *Lactococcus lactis* sub-sp. *lactis* biovar. *diacetylactis* (old nomenclature) and *Leuconostoc mesenteroides* sub-sp. *cremoris*; *Propionibacterium* spp. are employed in some Swiss cheeses as well; or

(ii) A thermophilic culture dominated by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus*, although species like *Lactobacillus helveticus*, *Lactobacillus casei* and *Lactobacillus acidophilus* are used quite widely; *Bifidobacterium* spp. are added to some 'health-promoting' fermented milks (38, 39). All these species are, (see Table 4) susceptible, to varying degrees, to inhibition by antibiotics or other compounds in milk, and hence the use of contaminated milk for cheese or yoghurt making may result in: (a) total vat failure and loss of the milk through the growth of adventitious bacteria resistant to the inhibitory substance(s); or (b) poor acid development and the risk of consumer complaints and/or incidents of food-borne disease (40).

Table 4. Typical minimum levels of penicillin G that will inhibit of some common lactic acid bacteria.

Genus	Level for Inhibition (IU/ml)
Lactococcus spp.	0.05
Lactobacillus bulgaricus	0.02
Stretococcus thermophilus	0.004
Yoghurt culture	0.01

Consequently, there can be little doubt that all dairies undertaking the fermentation of liquid milk need a system for monitoring supplies for inhibitory substances, and hence the aim of this study was to:

1. Devise a simple test for monitoring milk supplies, and one that needed only routine laboratory equipment; and

2. Ensure that the result would be available within 2.5 hours, so that unacceptable milks could be rejected before the start of processing.

MATERIALS AND METHODS

As shown in Table 4, the species used for the production of yoghurt tend to be the most sensitive of the lactic acid bacteria in common use. When growing together - as in a normal yoghurt culture - the sensitivy of the combination is slightly lower than that of the individual components, but it is still comparable the figures shown for some of the commercial kits. Consequently, it was decided to use *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus* in the Yoghurt Culture Test (YCT).

After preliminary screening of three cultures from the major Dairies in Jordan for their sensitivity to antibiotics, one was selected for the experimental programme. It was maintained by adding 1 g of a well mixed, fresh culture to 99 ml of skim-milk (10% total solids, w/v) which had been heat treated at 95°C for 5 min. After incubation at 42°C for 4 h, the culture was stored overnight at 4°C for use next day.

The sensitivity of the proposed test was measured using fresh, raw milk drawn from a single cow with no history of disease or antibiotic therapy. After cooling to 4°C, the pH of the milk was reduced to pH 6.0 using 1N HCl. The milk was then divided into seven portions, and the individual portions were dosed with a stock solution of penicillin G to give the following concentrations: 0.0 (control), 0.1, 0.06, 0.03, 0.006, 0.003 and 0.0006 IU/ml. The milks were then warmed in a microwave oven to 45°C, and each portion was inoculated with the yoghurt culture at a rate of 4% v/v; 1 ml/100 ml of Chlorophenol Red (0.2% in 50% ethanol) was added as an indicator. After thorough mixing, each portion of milk was distributed into ten test tubes (10 ml amounts), and the tubes were placed in a water bath at 42°C. Duplicate masurements of pH were made immediately using a Hanna Instruments pH Meter, and then after 1.5, 2.0, 2.5 and 4 h incubation.

The entire procedure was repeated with chloramphenicol (10.0 - 1.0 μg/ml of milk), oxytetracycline (0.50 - 0.05 μg/ml of milk), tetracycline (0.80 - 0.10 μg/ml of milk), ampicillin (0.20 - 0.05 μg/ml of milk), erythromycin (0.60 - 0.20 μg/ml of milk) and cloxacillin (0.60 - 0.20 μg/ml of milk).

Once the protocol had been established and the sensitivity of the

YCT against the different antibiotics confirmed, a full-scale field trial was undertaken. Over a period of three months, a total of 618 samples of milk from the three largest dairy plants in Jordan were tested with the YCT system. On each occasion, the dairy operator produced a composite sample from the milks of each supplier, and a 100 ml from this composite sample was transferred to a screw-capped bottle. The sample - stored in ice at 2 - 5°C, was returned to the laboratory within one hour and the pH measured.

Each sample of milk (around 100 ml) was then poured into an individual polyethylene yoghurt carton (200 g capacity), and sufficient 1N HCl added to give a pH of 6.0. Four grams of starter culture and 1.0 ml of Chlorophenol Red were added and, after mixing, the carton was placed in an incubator at 42°C. A control made up in antibiotic-free skim milk was run in parallel, and the colour change/curd formation was recorded at 2.5 and 4.0 h; samples without any change in colour/curd formation were suspected of being contaminated with antibiotics - positive. As an additional check, each of the samples that failed to form a coagulum after 2.5 or 4 h was tested with a Delvotest-P kit obtained from Gist-brocades, Delft, Holland, and used according to the manufacturer's instructions (31).

RESULTS AND DISCUSSION

The results of the preliminary trials showed that, with antibiotic-free milk of pH 6.0:

- the culture achieved a pH of 4.8 in 2.5 h;

- the chlorophenol red indicator changed colour from light violet to beige-yellow; and

- a soft coagulum had formed.

The minimum concentrations of the different antibiotics giving positive results in the YCT, ie. no curd formation or colour shift in the presence of Chlorophenol Red, are shown in Table 1.

The sensitivity of the test to pencillin was rather disappointing because, at 2.5 h, the YCT was ten times less sensitive than the published results for the Charm or Delvotest-P techniques. The reason for this difference may be that the *Lac. delbrueckii* sub-sp. *bulgaricus* component of the culture was not affected immediately by the inhibitor;

Table 1. Minimum concentrations of antibiotics (IU of penicillin or μg/ml) detected by the YCT as failures of the culture to lower the pH of the milk to 4.8 and form a coagulum within the time specified.

Antibiotic	Coagulum Formation (h)	
	2.5	4.0
Penicillin (IU)	0.05	0.10
	(0.03 and 0.06 μg/ml)	
Chloramphenicol	2.0	4.0
Oxytetracycline	0.1	0.25
Tetracycline	0.2	0.4
Ampicillin	0.1	0.15
Erythromycin	0.3	0.4
Cloxacillin	0.3	0.35

some strains of *Lac. delbrueckii* sub-sp. *bulgaricus* can tolerate 0.10 IU/ml of penicillin (39). It has been reported also that mixed cultures of *Str. thermophilus* and *Lac. delbrueckii* sub-sp. *bulgaricus* are less sensitive than the individual species growing alone, and this effect might have altered the results as well. The sensitivity of the YCT at 2.5 h was better for tetracycline, oxytetracycline, chloramphenicol and erythromycin than some reported figures for Delvotest-P (41), but the result for ampicillin revealed a lower sensitivity.

The lower sensitivity of the test at 4 h is probably a reflection of the fact that sufficient cells survive inhibition by the lower levels of antibiotic to reduce, albeit slowly, the pH of the milk to 4.8.

Nevertheless, based upon this performance, the following guidelines for monitoring the inhibitory effects of antibiotic substances were established.

1. Failure to change indicator in 2.5 and 4.0 h
Conclusion: unacceptable level of inhibitory substance(s) in the milk.

2. Failure to change indicator in 2.5, but change after 4.0 h
Conclusion: marginal level of inhibitory substance(s) in the milk.

3. Change of indicator in 2.5 h and coagulum formation
Conclusion: inhibitory substances below the level of detection.

This pattern is broadly comparable with that proposed for the Delvotest-P (31) for readings after 2.5 h incubation, that is:

(i) total colour change of the medium containing Bromocresol Purple from purple to yellow - residues of penicillin G < 0.002 IU/ml and comparable situation with other

inhibitory agents;
(ii) medium appears purple/green colour - concentration of penicillin G of 0.002 - 0.005 IU/ml; and
(iii) entire medium appears purple - concentration of pencillin G at least 0.005 IU/ml.

Clearly the disadvantage of the YCT is that it is not as sensitive to ß-lactam residues as some commercial kits, and hence Public Health Authorities operating a policy of 'zero tolerance' of antibiotics in dairy products may need to use another approach (27).

Nevertheless, the YCT was conceived as a reception test for dairies, and coagulation at 2.5 h will give a dairy total confidence to use the milk in a fermentation process. In addition, it is important that the YCT is simple, employs an in-house culture, needs little skill to perform and is inexpensive. Consequently, it was decided to use the YCT for a market survey of selected milk supplies in Jordan, with the Delvotest-P being employed to confirm any positive results.

One of the notable features of the survey was that care of the milk appeared to be variable in that, at the time of testing in the laboratory, 24% of the samples had a pH < 6.4 and 13% had a value < 6.2; 6% of the samples were below the target pH of 6.0 set for the YCT. These figures suggest that some of the milk is not being adequately cooled after collection and, for some processing procedures, these low values could cause problems.

The results of the antibiotic tests are shown in Table 2.

Table 2. Numbers of milk samples from three different dairy plants which gave positive results in the YCT, ie. no colour change and/or coagulation within the times indicated, and the numbers which were then confirmed by the Delvotest-P.

Source	Number		
	(a)	(b)	(c)
Dairy 1.	184	34	6(6)
Dairy 2.	311	29	7(7)
Dairy 3.	123	30	0(0)
Total	618	93	13(13)

(a) Total number of samples from each dairy
(b) Positives identified by YCT at 2.5 h; the numbers confirmed with Delvotest-P were 27, 21 and 28 for Dairies 1, 2 and 3, respectively.
(c) Positives indentified by YCT at 4.0 h; figures in brackets indicate confirmation with Delvotest-P.

It is notable that, when a sample failed the YCT after 4 h, ie. high levels of inhibitory substabces were present, the result of the Delvotest-P confirmed the result in all cases.

However, after incubation for 2.5 h, only 81% of the samples detected as positive' for inhibitory substances by the YCT were later confirmed as positive by the Delvotest-P. The importance of this latter result lies in the facts that:

(i) if the results of the Delvotest are taken as the 'standard', then the YCT at 2.5 h gave 19% 'false positives', ie. any 'failures' are on the side of caution. In other words, if a batch of milk does contain inhibitory substances, the dairy manager will be alerted early to a potential problem; on the basis of the result at 4 h, the manager can then decide to use the milk knowing that the process times may be extended slightly, or reject it if the result at 4 h remains positive;

(ii) the sensitivities to the two tests are not identical. For example, a failure with the YCT at 2.5 h could indicate inhibitory levels of sulphonamides, a group of antibiotic materials that are only detected by Delvotest-P at levels of 50 - 100 μg/ml; and

(iii) the YCT can be carried out with the culture that is employed in the dairy for the manufacture of yoghurt, so giving an direct indication of the suitability of the test milk for the process in question.

CONCLUSION

Overall, it would appear that a YCT employing selected strains of *Str. thermophilus* and *Lac. delbrueckii* sub-sp. *bulgaricus* could provide an extremely sensitive test for inhibitory substances in milk, and that a 'pass' at 2.5 h assures the manufacturer that the milk can be used in a fermentation process. Conversely, a 'failure' at 2.5 h highlights the risk of possible contamination and, although a 'failure' at 4 h is necessary to confirm that the milk should be rejected, the use of the YCT should be encouraged in countries where the testing of milk supplies for antibiotics is not mandatory.

Obviously, the workload on a dairy laboratory could become excessive in a country like Jordan where individual farmers deliver the milk direct to a dairy. However, in such situations, consideration might

be given to the establishment of collecting centres where milks could be tested, bulked and then delivered to dairy plants in the vicinity. Such an approach might serve to: (a) reduce the number of suppliers attempting to market contaminated milk - 2.1% of supplies identified in the survey contained unacceptable levels of inhibitory substances including, perhaps, antibiotics of the ß-lactam group; and (b) improve the chilled storage capacity in the area, and so avoid the build-up of acidity that was noted in many of the individual samples.

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FOOD CONTROL

A simple test for the detection of antibiotics and other chemical residues in ex-farm milk

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Abstract

A simple Yoghurt Culture Test (YCT) for the routine detection of antibiotic and other residues in raw milk was developed. It involved acidifying a sample of the milk to pH 6.0, adding Chlorophenol Red as an indicator and inoculating the milk (4%, v/v) with a culture containing a balanced mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus*. In the absence of inhibitory substances, incubation at 42°C for 2.5 h gave rise to sufficient acid production for the milk to gel and the indicator to change colour (PASS), but low levels of the antibiotics tested increased the incubation time beyond 4 h (FAILURE). A survey of 618 samples of raw milk collected from three dairies in Jordan identified a failure rate of 15% with the YCT at 2.5 h whereas, due to differences in sensitivity, the Delvotest-P failed only 12.3% of the milks. It was concluded that the YCT was a reliable and inexpensive approach for testing ex-farm milks for inhibitory residues.

Keywords: Milk; Antibiotics; Testing

1. Introduction

Antibiotics are essential for the control of mastitis and other bacterial problems in dairy herds (Jones and Seymour, 1988; Oliver et al., 1990), and their passage into milk can cause major problems for a receiving dairy. Thus, if the milk is to be processed for direct retail sale or into milk powder or some other product, it would be regarded in many countries as 'adulterated' and a public health risk (Albright et al., 1961). This intolerance of antibiotic residues stems from the fact that some adults are hypersensitive to penicillins, and allergic reactions and even death can result from ingestion (Seymour et al., 1988). In addition, any exposure of the intestinal microflora of humans to antibiotics may lead to an increase in the numbers of antibiotic-resistant species present and, if some of these are pathogenic, then their possible spread within a community could have dire consequences (McGrane et al., 1996). It is for this reason that the Federal Drug Agency in the USA have specified a 'zero tolerance' for antibiotics in milk and milk products, i.e. no higher than the lowest concentration that can be detected by prevailing techniques (Kornfield, 1977; IDF, 1991).

For the manufacturers of cheese or fermented milks, the situation is slightly different, for the presence of antibiotics or other inhibitory materials in milk can lead to total or partial failure of the starter culture. If the failure is total, then an entire vat of milk may be wasted, while if acid development is slow or inadequate, consumer complaints may arise if poor product reaches the market or, in the case of certain cheeses, there may be a risk of a serious incident of food-borne disease (Keceli and Robinson, 1997). In order to avoid the financial losses that may be associated with the use of contaminated milk, both the dairy industry and Regulatory Authorities have come to rely on routine programmes of testing (Shearer, 1995; Robinson and Wilbey, 1998). For this purpose, a number of microbial inhibitor tests approved by the AOAC (1990) or other Bodies are available, such as the Cylinder Plate Assay

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and the Disc Assay(s) (Marshall, 1992), the Delvotest-P and Delvotest-SP (Anon, 1997), and the Charm AIM-96 Test (Marshall, 1992; McGrane et al., 1996; Suhren and Heeschen, 1996). In general, these broad spectrum tests take > 2.5 h to complete but, more recently, a number of rapid (< 10 min) ELISA systems have been introduced which are highly specific for β-lactam residues (Bell et al., 1995; Scannella et al., 1997). In the case of penicillin G, most of these procedures can detect between 0.004 and 0.006 IU ml^{-1} of milk, but the responses of the microbial inhibitor tests to other antibiotics or inhibitory residues varies with the compound in question. As far as the fermentation sector of the dairy industry is concerned, these figures compare favourably with the known sensitivity of *Streptococcus thermophilus* (Tamime and Deeth, 1980), a species that is amongst the most susceptible of starter bacteria to inhibition.

In Europe, North America and Australasia, dairy factories can, therefore, monitor their milk supplies on a routine basis and, given the speed of the ELISA tests, are in a position to reject contaminated tanker loads in advance of discharge into a silo. However, this use of an antibiotic residues test as a 'reception test' is a comparatively recent innovation and, as most recognised tests take > 2.5 h, their use is intended, at least in part, to avoid vat failure through the prior isolation of any heavily contaminated supplies. In the less developed parts of the world, the non-availability of imported commercial kits and the cost of each test tends to preclude even this essential monitoring (Jurdi and Asmar, 1981). Nevertheless, these restrictions do not mean that testing should not be implemented, and hence the aim of this project was to:

(i) examine the possibility of employing a simple, standardised growth test to check milk supplies for inhibitory materials within the same time-span as the widely used microbial inhibitor tests; and
(ii) compare the performance of the test against an internationally-accepted method — the Delvotest-P.

2. Materials and methods

Three yoghurt cultures — each containing equal mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus* — that are in regular use in dairy plants in Jordan were selected for the project. Each culture was prepared by mixing 1 g of well-mixed, fresh yoghurt with 99 ml of skim-milk (10% dry milk solids, w/v) that had been heat treated at 95°C for 5 min. After incubation at 42°C for 4 h, the cultures were stored at 5°C. Fresh sub-cultures were prepared in the same manner, and were always used on the following day.

2.1. Establishment of the procedure

Fresh, raw milk was drawn from a single cow known to be free from any form of medication, and the sample was transported to the laboratory in an ice-box; the maximum journey time was around 1 h. Appropriate volumes of the milk were then dispensed into four clean jars and, while one jar was held as a control, the pHs of the other samples were adjusted to 6.4, 6.2 and 6.0 using 1 N HCl. The milk in each jar was then divided into four equal portions and, after warming in a domestic microwave oven (Toshiba ER 692) for a period of time known to give a temperature of 45°C, the four portions were inoculated with either 2, 3, 4, or 5% (w/w) of one of the three available cultures. This procedure was repeated for each pH treatment and control, and with each of the three cultures. After thorough mixing, 10 ml aliquots from each portion were transferred to each of 10 test tubes, and the tubes placed in a water bath at 42°C. Duplicate measurements of pH were made immediately using a Hana Instruments pH meter (Model HI 8416), and after 1.5, 2.0 and 2.5 h incubation; the titratable acidities of the same samples were measured using the procedure of Case et al. (1985).

As an alternative to measurements of acidity, the use of pH indicators was examined in a further trial by adding 0.1 ml of either Bromocresol Green (0.2% in 50% ethanol), Methyl Red (0.2% in 50% ethanol), Chlorophenol Red (0.2% in 50% ethanol) or Bromocresol Purple (0.2% in 50% ethanol) to the milk either before or after incubation.

2.2. Sensitivity of the test to different antibiotics

Stock solutions of each antibiotic, namely penicillin, chloramphenicol, oxytetracycline, tetracycline, ampicillin, erythromycin and cloxacillin, were prepared in deionised water at 10 times the maximum concentration to be tested. Appropriate aliquots were then added to standard volumes of fresh, raw cow's milk to give the following concentrations:

Penicillin — 0.1, 0.06, 0.03, 0.006, 0.003 and 0.0006 IU ml^{-1} of milk
Chloramphenicol — 10.0, 5.0, 4.0, 3.0, 2.0 and 1.0 μg ml^{-1} of milk
Oxytetracycline — 0.50, 0.25, 0.20, 0.15, 0.10 and 0.05 μg ml^{-1} of milk
Tetracycline — 0.80, 0.60, 0.40, 0.30, 0.20 and 0.10 μg ml^{-1} of milk
Ampicillin — 0.20, 0.15, 0.10, 0.075 and 0.05 μg ml^{-1} of milk

Erythromycin — 0.60, 0.50, 0.40, 0.30 and 0.20 $\mu g\ ml^{-1}$ of milk
Cloxacillin — 0.40, 0.35, 0.30, 0.25 and 0.20 $\mu g\ ml^{-1}$ of milk

The pH value of each sample of milk was then adjusted to 6.0 with 1 N HCl, and the sample warmed to 45°C in the microwave. A fresh starter culture was then used to inoculate the contaminated milks, along with a control sample (zero antibiotic), at a rate of 4%, and Chlorophenol Red was added as an indicator. After mixing well, 10 ml amounts of each milk were dispensed into test tubes, and the tubes incubated at 42°C. Measurements of pH and acidity were made at 0, 1.5, 2.0, 2.5 and 4.0 h, and the colour changes were recorded as well.

2.3. Market test of the YCT procedure

Over a period of 3 months, a total of 618 samples of milk supplied to three largest dairy plants in Jordan were examined using the Yoghurt Culture Test (YCT). On each occasion, the dairy operator produced a composite sample from the milks of each supplier, and 100 ml from this composite sample were transferred to a screw-capped bottle. The sample, stored in ice at 2–5°C, was returned to the laboratory within 1 h and the pH measured.

Each sample of milk (around 100 ml) was then poured into an individual polyethylene yoghurt carton (200 g capacity), and sufficient 1 N HCl added to give a pH of 6.0. Four grams of starter culture and 1.0 ml of Chlorophenol Red were added and, after mixing, the carton was placed in an incubator at 42°C. A control made up in antibiotic-free skim milk was run in parallel, and the colour change/curd formation was recorded at 2.5 and 4.0 h; samples without any change in colour/curd formation were suspected of being contaminated with antibiotics and were considered positive. As an additional check, each of the samples that failed to form a coagulum after 2.5 or 4 h was tested with a Delvotest-P kit obtained from Gist-brocades, Delft, The Netherlands, and ampoules that were entirely or partially purple at the end of 2.5 h were recorded as positive.

3. Results and discussion

The preliminary trials confirmed that the optimum conditions to achieve curd formation — pH 4.8 and titratable acidity of 0.5% (as lactic acid) — within 2.5 h involved an inoculation rate of 4.0% and an initial pH of the milk of 6.0. However, only one of the three starter cultures was able to achieve this level of performance on a repeatable basis, and hence the other two were eliminated from the trial. It was found also that Chlorophenol Red was the most appropriate indicator, as the change in colour from light violet to beige–yellow was easy to note during routine observations.

Table 1
Minimum concentrations of antibiotics detected by the YCT as measured by the time for the culture to lower the pH of the milk to 4.8, i.e. achieve minimum coagulum formation

Antibiotic	Coagulum formation ($\mu g\ ml^{-1}$)	
	2.5 h	4.0 h
Penicillin	0.03 (0.05 IU)	0.06 (0.1 IU)
Chloramphenicol	2.0	4.0
Oxytetracycline	0.1	0.25
Tetracycline	0.2	0.4
Ampicillin	0.1	0.15
Erythromycin	0.3	0.4
Cloxacillin	0.3	0.35

The minimum concentrations of the different antibiotics giving positive results in the YCT, i.e. no curd formation or colour shift in the presence of Chlorophenol Red, are shown in Table 1. The sensitivity of the test to penicillin was rather disappointing because, at 2.5 h, the YCT appeared 10 times less sensitive than some of the results published for the Charm or Delvotest-P techniques, and it may be that the *Lac. delbrueckii* sub-sp. *bulgaricus* component of the culture was not affected immediately by the inhibitor; Tamime and Deeth (1980) suggest that some strains of *Lac. delbrueckii* sub-sp. *bulgaricus* can tolerate 0.10 IU ml^{-1} of penicillin. It has also been reported that mixed cultures of *Str. thermophilus* and *Lac. delbrueckii* sub-sp. *bulgaricus* are less sensitive than the individual species growing alone, and this effect might have altered the results as well (Robinson and Tamime, 1990). The sensitivity of the YCT to chloramphenicol, oxytetracycline, tetracycline and erythromycin at 2.5 h was better than that reported for Delvotest-P (Jones and Seymour, 1988), but the result for ampicillin revealed a lower sensitivity.

The reduced sensitivities of the YCT at 4 h is a reflection of the fact that the concentrations that cause a 'failure' at 2.5 h leave a percentage of cells of one or both organisms unaffected. Consequently, sufficient acidity has been generated at the end of 4 h to form a coagulum, and a higher concentration is needed to ensure that too few cells survive to lower the pH to 4.8 or below. Nevertheless, the results at 4 h were useful for developing the following protocol:

Failure to change indicator in 2.5 and 4 h
— unacceptable level of inhibitory substances in the milk

Failure to change indicator in 2.5 h, but change after 4 h
— marginal level of inhibitory substances in the milk
Change of indicator in 2.5 h
— inhibitory substances below level of detection

This pattern is broadly comparable with that proposed for detection of penicillin G with the Delvotest-P (Anon, 1997) with readings after 2.5 h incubation, i.e. (i) total colour change of the medium containing Bromocresol Purple from purple to yellow — residues of penicillin G <0.002 IU ml^{-1} and comparable situation with other inhibitory agents; (ii) medium appears purple/green colour — concentration of penicillin G of 0.002–0.005 IU ml^{-1}; and (iii) entire medium appears purple — concentration of penicillin G >0.005 IU ml^{-1}.

Clearly, the disadvantage of the YCT is that it is not so sensitive to β-lactam antibiotics as some of the commercial kits, but this criticism does not alter the value of the YCT as a practical method of assessment for a dairy. In other words, as long as the working culture can coagulate the milk in 2.5 h, the dairy can use the milk with confidence for a fermentation process, whereas, if the milk fails at 4 h, it is totally unsuitable for a fermentation process. Whether or not the 'marginal' category is helpful is open to debate, but at least the result alerts the dairyman to an impending problem. In addition, the YCT is simple, employs an in-house culture, needs little skill to perform and is inexpensive, so that its use as a routine screen of supplies could find wide application. Consequently, it was decided to use the YCT for a market survey of selected supplies of raw milk in Jordan, with the Delvotest-P being employed to confirm any positive results; it was assumed that negative results would be negative with the Delvotest-P as well.

One of the notable features of the survey was that care of the milk appeared to be variable, in that, at the time of testing in the laboratory, 24% of the samples had a pH <6.4 and 13% had a value <6.2; 6% of the samples were below the target pH of 6.0 set for the YCT. These figures suggest that some of the milk is not being adequately cooled after collection and, for some processing procedures, these low values could cause problems.

The results of the antibiotic tests are shown in Table 2, and it is important that, whereas after incubation for 2.5 h only 81% of the samples detected as 'positive' for inhibitory substances by the YCT were later confirmed as 'positive' by the Delvotest-P, samples that failed the YCT after 4 h were all confirmed as containing a high level of one or more inhibitory substances by the Delvotest-P. The practical significance of this pattern lies in the facts that:

(i) if it is assumed that the results of the Delvotest-P were 'correct', then the YCT at 2.5 h gave 19% 'false positives', i.e. any 'failures' were on the side of caution;
(ii) the sensitivities to the two tests are not identical. For example, a failure with the YCT at 2.5 h could indicate the presence of inhibitory levels of sulphonamides, a group of antibiotic materials that are only detected by Delvotest-P at levels of 50–100 μg ml^{-1}. Thus, it could be that the results referred to above as 'false positives' could, in reality, be providing a true reflection of the quality of the milk; and
(iii) the YCT can be carried out with the culture that is employed in the dairy for the manufacture of yoghurt, so that the result at 2.5 h gives an immediate indication of the suitability of the test milk for processing.

Overall, it would appear that the YCT employing sensitive strains of *Str. thermophilus* and *Lac. delbrueckii* sub-sp. *bulgaricus* provides a test for inhibitory substances in milk that is broadly comparable in response to the Delvotest-P. Obviously, the YCT would not be suitable for use in the laboratory of a Regulatory Authority where the priority is to protect consumers from extremely low levels of β-lactam residues, but use of the YCT could be encouraged in countries where the testing of milk supplies for antibiotics is not mandatory.

Table 2
Numbers of milk samples from three different dairy plants which gave positive results in the YCT, i.e. no colour change and/or coagulation within the times indicated, and the numbers which were then confirmed by the Delvotest-P

Source	Number	Positives identified by YCT		Positives identified by YCT and confirmed by Delvotest-P	
		at 2.5 h	at 4 h	at 2.5 h	at 4 h
Dairy 1.	184	34	6	27	6
Dairy 2.	311	29	7	21	7
Dairy 3.	123	30	0	28	0
Total	618	93	13	76	13

What emerged also from the survey was that the workload on a dairy laboratory could become excessive in a country like Jordan where individual farmers deliver the milk direct to a dairy, and consideration should, perhaps, be given to the establishment of collecting centres where milks could be tested, bulked and then delivered to dairy plants in the vicinity. The attraction of such an approach is that it might serve to: (a) reduce the number of farmers attempting to market contaminated milk — 2.1% of supplies tested in the survey contained unacceptable levels of inhibitory substances, including probably antibiotics of the β-lactam group; and (b) improve the chilled storage capacity in the area, and so avoid the build-up of acidity that was noted in many of the individual samples.

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The effect of elevated milk solids and incubation temperature on the physical properties of natural yoghurt

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1. Introduction

It is well known that raising the level of solids-non-fat (SNF) in bovine milk above the typical value of 8.5–9.0% improves the gel strength of set yoghurt and/or the viscosity of the stirred product. The values for these physical parameters tend to increase with levels of non-fat solids up to a maximum of 16% (1), but the benefits of exceeding this figure are usually marginal – at least as measured by the penetrometer or viscometer. It has been argued that the increase in gel strength arises from an increase in the number of protein-protein bonds that can occur at elevated solids levels, but there is some controversy as to the extent to which the method of fortification, *e.g.* addition of skim-milk powder, vacuum evaporation or membrane processing, of the process milk affects the situation.

The viscosity of stirred yoghurt may be affected in a rather different manner, but for both set and stirred yoghurts, the characteristics of the starter culture and the conditions of incubation can influence the properties of the end-product. One of the problems in assessing any responses to process parameters is that the usual methods of physical measurement involve a destruction of the gel structure (2).

Thus, yoghurt has a weak gel structure formed from a 3-dimensional network which immobilises the liquid phase (3), and any disturbance breaks the chains of casein micelles. However, by examining such a structure with a controlled-stress rheometer, 2 parameters which indicate the elastic and viscous characteristics of the gel can be determined. Firstly, the storage modulus G' is measured, which expresses the stored energy in the material from the rearrangements in the structure which can take place during the oscillation period; solids tend to return to the original state after a stress is released, and hence G' confirms the elastic characteristics of the product. Then, secondly, the loss modulus G", which records the energy lost during the cycle of deformation – so indicating the viscous component of the material, is recorded. These measurements should, therefore, give an accurate picture of the gel structure and any changes that occur during stirring, but it has yet to be demonstrated convincingly that the results from the application of destructive techniques are in any way misleading with respect to the physical properties of yoghurt. In addition, rheometry is a technically-demanding procedure compared with the use of the conventional penetrometer or viscometer, so that the aims of this work were:

a) to produce natural set and stirred yoghurts with 16% SNF, with the elevated total solids being achieved by the addition of skim-milk powder, concentration by ultrafiltration or concentration by vacuum evaporation;
b) to evaluate the gel strength (set yoghurt) and viscosity (stirred yoghurt) by both destructive and non-destructive techniques;
c) using a non-polysaccharide producing culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, assess the impact of incubation temperature on the above properties; and
d) compare the data obtained by the alternative techniques, and consider their potential as a basis for comparing the physical quality of the yoghurts made from the 3 types of milk.

2. Materials and methods

2.1 Production of the yoghurts

A batch of pasteurised liquid skim-milk (8.5% SNF) was divided into 3, and the individual portions were treated as follows:

a) addition of skim-milk powder (SMP) (Adams Food Ingredients, Leek, Staffs, England) to give a final total solids level of 16%;
b) concentration at –0.6 bar and 57–60°C through a climbing-film evaporator (VE) (Corning Process Systems, Stone, Staffs, England) to 16% total solids; and
c) multiple passes at 45°C through a tubular PCI Ultrafiltration (UF) Unit (PCI Membranes, Whitchurch, Hants,UK) using a polyether sulphone (model ES 625) membrane with an area of 0.8 m², a MWt cut-off of 25,000 Daltons, and inlet and outlet pressures of 0.6 and 0.3 MPa, respectively, until the total solids content was 16%.

The individual batches were then heat-treated at 85°C for 30 min, prior to cooling to 30°C for inoculation (2% v/v) with a culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The starter culture had been grown in sterile skim-milk (12% total solids) with incubation at 42°C to give a pH of 4.7–4.8 and a ratio between the genera of 1:1 'clump count' (1). After stirring the inoculated milk for 10 min to distribute the culture, 1 portion of each batch was poured into polyethylene cartons (175 ml in 200 ml cartons), whilst the remainder was divided between 2 stainless steel beakers (1,750 ml in each 2 l beaker). One beaker was placed in an incubator at 42°C until the pH reached 4.3, at which point the yoghurt was transferred to a cold-store at 4°C for 24 h. The other beaker (1,750 ml of inoculated milk) was placed immediately in a cold-store at 4°C, and then was later placed in an incubator at 30°C for an overnight fermentation to pH 4.3; this yoghurt was also held at 4°C for 24 h before examination.

In the cases of the set yoghurts, the cartons were incubated at either 42 or 30°C to pH 4.3, and again a pe-

riod of 24 h at 4°C was allowed prior to assessment of gel strength.

2.2 Assessment of the physical properties

The gel strength was measured with a standard penetrometer (Stanhope Seta Ltd., Camberley, Surrey, UK) using a probe of 67 g, a diameter of 2.5 cm and an apical angle of 90°; the penetration time was 5 s. In all cases, the temperature of the yoghurt was equilibrated at 25°C to match the temperature of the sample being studied with the rheometer, and duplicates pots from each batch were assessed.

The bulk samples of yoghurt were stirred manually for 5 min to achieve a visually homogeneous product and, after standardising the temperature at 25°C, the apparent viscosity was measured with a Brookfield Viscometer (Model LVT with Helipath Attachment) (Brookfield Engineering Inc., Stoughton, USA) fitted with a T-bar spindle (D) rotating at 0.6 rotations/min; the readings were converted to centipoises using the factor supplied by the manufacturer. For both gel strength and viscosity measurements, the samples were stored for 18 days to simulate a typical shelf-life in a retail situation.

A Rheotech International Controlled-Stress Rheometer (Camtel Ltd., Royston, Herts, UK) was used to make the dynamic measurements, and the moving surface was a parallel plate of 20 mm diameter; the gap between this plate and the stationary surface was 10 mm. To establish the conditions for set and stirred yoghurt, the frequency and amplitude ranges were tested to find the so-called linear viscoelastic region (LVE) (4). In this region, both shear moduli are independent of strain and stress, and it ends at the same applied strain where the material begins to break down. The experimental conditions were established as:

Yoghurt	Frequency	Amplitude (Minimum)	Amplitude (Maximum)
Set	0,25 Hz	0,015 mN m	0,15 mN m
Stirred	0,25 Hz	0,008 mN m	0,08 mN m

All measurements were made at 25°C.

Before all assessments, the yoghurts were held at 25°C in a constant temperature room to equilibrate, and were stirred by means of a low speed stirrer (speed setting 63 rpm, stirring time 5 s, diameter of stirrer vessel = 10 cm) in order to standardise the condition of the samples. Each test specimen was allowed to relax (5 min) prior to assessment of its amplitude behaviour. The 3 types of yoghurt were examined after 11 days to simulate the mid-point of a typical shelf-life.

3. Results and discussion

Samples of set and stirred yoghurts made from milks concentrated/fortified by different techniques and incubated at either 30 or 42°C were analysed in terms of their physical properties. The values for gel firmness of the set yoghurts after 18 d storage at 4°C are shown in Fig. 1, while the viscosity measurements for the stirred yoghurts are shown in Fig. 2.

Overall, viscosity and penetrometer values were higher for UF-treated sample irrespective of incubation temperature, but the differences between SMP-fortified yoghurts and those made from vacuum-evaporated milk

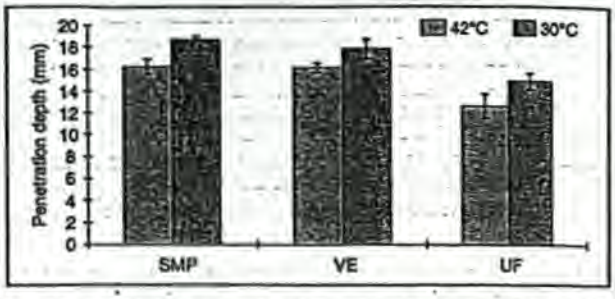

Fig. 1: Apparent gel strength of natural set yoghurts of 16% total solids achieved by adding skim-milk powder (SMP) to liquid milk (8.5% total solids), or concentrating the milk by vacuum evaporation (VE) or ultrafiltration (UF); all samples stored for 18 d at 4°C. Measurements, as depth of penetration (mm) of a standard cone/spindle at 25°C, are the means of 3 readings

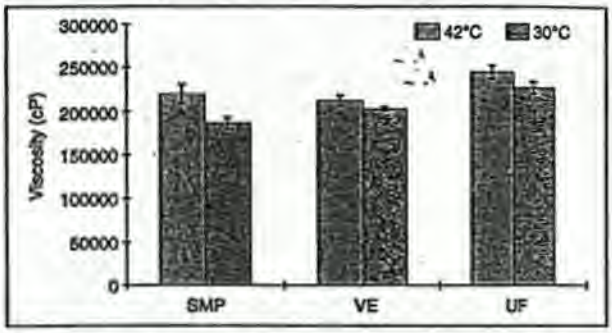

Fig. 2: Apparent viscosities of natural stirred yoghurts of 16% total solids achieved by adding skim-milk powder (SMP) to liquid milk (8.5% total solids), or concentrating the milk by vacuum evaporation (VE) or ultrafiltration (UF); all samples stored for 18 d at 4°C. Measurements, as viscosities (centipoises) recorded with standard Brookfield Viscometer at 25°C, are the means of 3 readings

were relatively small. This contrast with the UF samples could be due to differences in the protein contents of the yoghurts for, with the application of UF application, only high molecular weight compounds were detained in the retentate so that, at the same levels of total solids, the UF sample had the highest level of protein; in VE and SMP-added samples, all the milk components increased proportionally. As a higher protein content leads to a higher number of protein interactions and protein-protein bonds, so the elastic character of the gel increases as shown in Fig. 1. The same increase in concentration of protein causes the space occupied by the protein network to increase, so restricting the mobility of free water and higher values for viscosity (Fig. 2).

In all samples, although the trends with respect to the physical characteristics remained unchanged, the recorded values declined when the incubation temperature was lowered to 30 from 42°C. As expected, the retarded activity of the thermophilic starter resulted in a slower rate of acidification – the incubation periods for 42 and 30°C were 4–5 and 14–15 h, respectively, and this slow gelation may have led to a difference in the kinetics of protein network formation. Thus, at low temperatures, the activation energy (Helmholtz energy) required for gelation (formation of physically-active protein bonds) increases and *vice versa* (5), and a minimum

Helmholtz activation energy is essential for the formation of an irreversible gel network; a decrease in Helmholtz activation energy causes faster gelation, especially during the early stages of incubation.

In all the systems, the number and distribution of both the strong and weak (reversible) bonds increased with incubation temperature, a conclusion confirmed by the higher values for storage (G') and loss (G") moduli (Figs. 3 and 4, respectively). It is fair to assume that the stronger protein bonds contribute to the elastic character of viscoelastic gels, whereas the loss modulus (G") reflects the number and/or distribution of weak bonds. Both, the number and distribution of protein bonds throughout the gel network seems to be dependent on the protein content as well, for the UF samples gave higher storage moduli than the others.

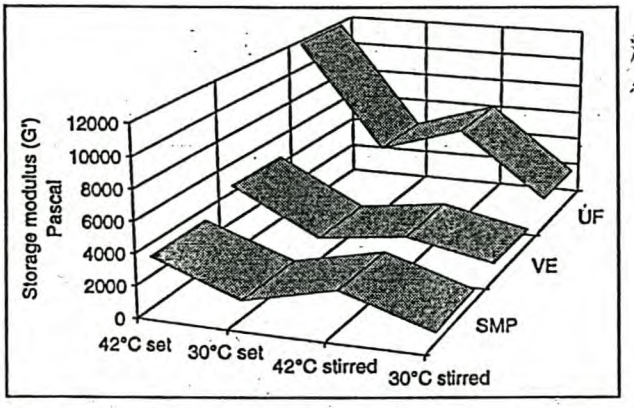

Fig. 3: Storage moduli (G') of natural set and stirred yoghurts of 16% total solids achieved by adding skim-milk (SMP) to liquid milk (8.5% total solids), or concentrating the milk by vacuum evaporation (VE) or ultrafiltration (UF); all samples stored for 11 d at 4°C. All measurements were made at 25°C, and are the means of 3 readings

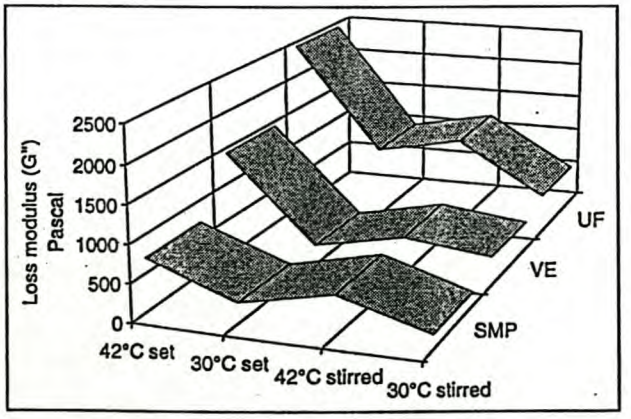

Fig. 4: Loss moduli (G") of natural set and stirred yoghurts of 16% total solids achieved by adding skim-milk (SMP) to liquid milk (8.5% total solids), or concentrating the milk by vacuum evaporation (VE) or ultrafiltration (UF); all samples stored for 11 d at 4°C. All measurements were made at 25°C, and are the means of 3 readings

However, the loss tangent (tan δ) values remained unaffected by incubation temperature or technique of processing, so suggesting that the nature of the interactive forces were essentially independent of the variables examined (Fig. 5); the loss tangent (tan δ = G"/G') is indicative of the nature of the interaction forces in a gel (5).

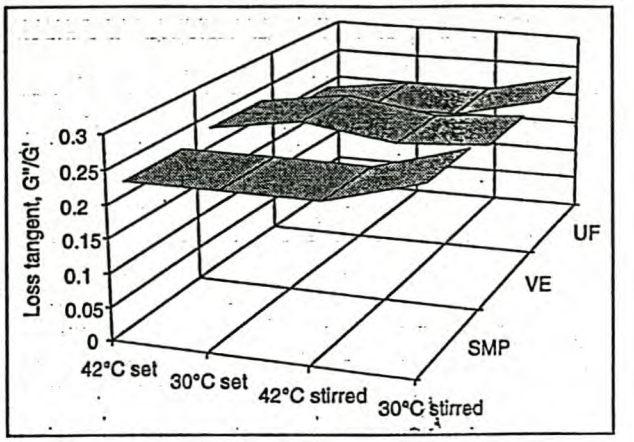

Fig. 5: Loss tangent (tan δ) of natural set and stirred yoghurts of 16 % total solids achieved by adding skim-milk (SMP) to liquid milk (8.5% total solids), or concentrating the milk by vacuum evaporation (VE) or ultrafiltration (UF); all samples stored for 11 d at 4°C. All measurements were made at 25°C, and are the means of 3 readings

It was notable also that the results of the dynamic (small deformation/non-destructive) rheological tests mirrored physical properties (at both incubation temperatures) observed by the destructive techniques (viscometer and penetrometer). This apparent correlation appears to contradict the earlier findings of OZER (2) who failed to find any agreement between small and large deformation tests using reconstituted whole milks concentrated by ultra-filtration or reverse osmosis. Whether contrasted starting materials or differences in the test parameters were responsible for this conflict was not established but, in the present context, the traditional techniques appeared to provide a good indication of the rheological properties of the yoghurts.

4. Conclusions

Although the differences in gel strength and viscosity between the UF and VE yoghurts were not extreme, the dynamic meaurements made it clear that UF concentration of the process milk is a suitable technique for the manufacture of yoghurt. Whether the heat treatment under vacuum evaporation caused the milk proteins to be less reactive during the yoghurt making process was not established, but the UF milk did produce yoghurts with firmer and more viscous structures. For small producers wishing to change from SMP fortification, UF offers a further advantage over VE in terms of plant flexibility, for the economics of vacuum evaporation tend to favour large throughputs of milk.

It was evident also that the higher incubation temperature (42°C) should be employed to achieve both the optimum rate of acidification and the minimum requirement for activation energy (Helmholtz energy), and it might be interesting to investigate this aspect of gelation further.

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6. Summary

LANKES, H., OZER, H.B., ROBINSON, R.K.: **The effect of elevated milk solids and incubation temperature on the physical properties of natural yoghurt.** Milchwissenschaft 53 (9) 510–513 (1998).

61 **Yoghurt** (physical properties)

Liquid skimmed milk (8.5% total solids (TS)) concentrated to 16% TS by ultra-filtration gave better quality yoghurt with respect to gel strength (set yoghurt) or viscosity (stirred yoghurt) than milk fortified to the same extent with skim-milk powder or reduced in volume by vacuum evaporation to 16% TS. This contrast was confirmed both by techniques destructive to the gel structure (penetrometer/set yoghurt and viscometer/stirred yoghurt) and by non-destructive dynamic rheometry. Although the precise reasons for the beneficial effects of ultra-filtration were not explored, the findings could have important implications for small-scale producers wishing to invest in process plant to avoid the use of expensive skim-milk powder.

LANKES, H., OZER, H.B., ROBINSON, R.K.: **Wirkung von erhöhter Milchtrockenmasse und Inkubationstemperatur auf die physikalischen Eigenschaften von Naturjoghurt.** Milchwissenschaft 53 (9) 510–513 (1998).

61 **Joghurt** (physikalische Eigenschaften)

Flüssige Magermilch (8,5% Trockenmasse (TM)) konzentriert auf 16% TM durch Ultrafiltration ergab einen qualitativ besseren Joghurt hinsichtlich Gelfestigkeit (fester Joghurt) oder Viskosität (gerührter Joghurt) als Milch, die mit Magermilchpulver versetzt oder im Volumen durch Vakuumevaporation auf 16% TM konzentriert worden war. Dieser Gegensatz wurde durch die Gelstruktur „zerstörende" Verfahren (Penetrometer/Setzjoghurt und Viskosimeter/Rührjoghurt) und durch nicht-destruktive dynamische Rheometrie bestätigt. Obwohl die genauen Gründe für den positiven Effekt der Ultrafiltration nicht untersucht wurden, könnten die Befunde wichtige Auswirkungen für kleinere Betriebe haben, die in die Verarbeitungsanlagen investieren wollen, um die Verwendung von teurem Magermilchpulver zu vermeiden.

7

The potential of inulin as a functional ingredient

Richard K. Robinson

Examines the behaviour of inulin in foodstuffs

A functional food is now defined as one that has an enhanced physiological effect[1], and it is no surprise that yogurts and/or bio-yogurts fall into this category. The beneficial features of these products are normally associated with the bio-availability of minerals or the properties of the starter culture for, whether the latter organisms survive digestion and become implanted[2], or merely act as a source of enzymes and vitamins following autolysis[3], the benefits are well documented.

In normal yogurt, various attempts have been made to enhance these functional properties further by including sources of dietary fibre/non-digestible carbohydrates but, needless to say, such end-products tend to have limited organoleptic appeal. Nevertheless, few people doubt the wisdom of consuming crude fibre on a regular basis to improve the transit of waste materials through the colon[4], or that other non-digestible plant materials can be important components of the diet of adult humans. The oligosaccharides are one category of these non-digestible carbohydrates that could prove attractive as functional ingredients[5], and inulin, in particular, could be of especial interest to the dairy industry.

Properties of inulin

Inulin consists of linear chains of fructosyl units joined by β(2-1) bonds, and it is this bonding that renders it resistant to hydrolysis, both in the stomach and the intestine[6]. It passes, therefore, directly to the colon where it is fermented by the bifidobacteria[7]. This group of bacteria are essential for the healthy functioning of the colon, and some of the reported benefits of high population levels in the intestine are cited by Kurmann and Rasic[8]. More specifically, species of *Bifidobacterium* act in the colon in a number of ways:

- They metabolize carbohydrate materials with the release of lactic and acetic acids, both of which serve to lower the pH of the colonic contents and suppress the activities of putrefactive bacteria. Acetic acid is the most effective in this respect, and it may well not only prevent overgrowth of the walls of the colon by undesirable bacteria – an occasional side-effect of ME influenza, for example – but also lower the risk of potentially carcinogenic compounds accumulating[2].
- The bacteria attain a high population level along the walls of the colon, and degrade the mucin secreted naturally by the epithelial cells; although the secretion of mucin is essential, excessive levels can lead to diarrhoea.
- They may stimulate the immune system of the host and prevent invasion by pathogenic bacteria.

In essence, the colon cannot function correctly without a microflora dominated by *Bifidobacterium* spp. and, bearing in mind that the population levels can be easily depleted by a range of adverse factors ranging from simple ageing through to antibiotic and/or radiation therapy, any natural means of reinforcing their presence must be desirable. The question arises, therefore, could the inclusion of inulin in natural or fruit yogurt provide the same benefit to the consumer as a "bio-yogurt" incorporating bifidobacteria? Obviously, other foods could be considered as vehicles for inulin, but yogurt would appear to be a sound choice in that, while the short chain lengths of the inulin should not interfere too much with the inter-micellar bonding of the casein, they should still have the capacity to impart a degree of viscosity to the whey phase of the product. Consequently, the study reported here sought to establish:

- the optimum level of casein necessary to give maximum gel strength to a natural yogurt as a result of protein-protein interactions;
- the effect of diffferent levels of oligosaccharide on lactic acid production by a standard yogurt culture – a bioculture including *Bifidobacterium* sp. might utilize some of the inulin to generate undesirable levels of ethanoic acid; and
- the influence of inulin on both the gel strength, viscosity and flavour of natural yogurt.

British Food Journal. Vol. 97 No. 4, 1995, pp. 30-32

Materials and methods

Skimmed-milk powder was reconstituted to give batches of milk containing either 12, 14, 16, 18 or 20 per cent total solids, which gave a range of protein (casein) values (by calculation) of 3.6-6.0 per cent. Each batch was heat-treated at 85°C for 30 minutes, before being cooled to 42°C and inoculated with a liquid culture (2 per cent v/v) of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus* in the ratio of 1:1 (clump: clump). The milks were then dispensed as 400ml aliquots into polypropylene containers with press-on lids, and the cartons incubated at 42°C. The pH of each batch was monitored hourly until the "control" sample reached pH 4.2 and, at this point, the remaining cartons were placed in a refrigerator at 4°C.

Next morning, a penetrometer was employed to test the istance of the protein networks to a standard cone and spindle (125g/apical angle – 45°), for it is reasonable to assume that the greater the depth of penetration of the spindle, the lower the degree of protein-protein bonding. Once the measurements of gel strength had been obtained, each batch of yogurt was transferred to a slow-speed mixer and blended to an even consistency for the viscosity to be recorded with a Heli-path Viscometer (Brookfield LVT) and T-spindle.

The initial trials showed that a total solids level of 16 per cent (4.8 per cent casein) gave a degree of protein bonding close to the maximum – at least as measured by the technique in question, and this base was used, therefore, for the incorporation of inulin at levels of 1, 5 and 10 per cent. These levels were selected on the basis of the usual dietary values for inulin (2-12g per person per day in Europe[5], so that, even at a rate of addition of 10 per cent, the consumption of one carton of yogurt (125g) on any en day would not lead to an excessive intake; the maximum dietary intake per day tends to peak at around 20g. The heat-treatment of the milk and the production of the yogurt was completed as described above, and the measurements of gel strength and viscosity were completed after overnight cooling.

Results and discussion

The penetrometer readings for the yogurts with different total solids are shown in Table I, and it is clear that, beyond a level of 4.8-5.0 per cent casein, the configuration of the micellar structure is such that additional bonding is unlikely to occur. Indeed, the typical structure of the coagulum, as revealed in Plate 1, suggests that whey-filled interstices will always limit the degree of aggregation[9].

Given these basic properties, it was anticipated, extrapolating from previous work carried out with polysaccharides[10,11], that the incorporation of inulin would:

Table I. *Depth of penetration over a period of ten seconds of a standard cone and spindle into set yogurts of different total solids at 5°C*

Total solids (%)	Depth of penetration (mm)
12	37.5
14	23.0
16	20.0
18	19.2
20	17.7

Note: All readings are the means of four samples

- interfere with the extent of bonding to give a decrease in gel strength in proportion to the concentration of oligosaccharide; and
- give an increase in viscosity as the concentration of inulin in the whey phase rose.

However, the figures in Table II appear to show a rather different pattern, in that the mean readings for gel strength can be assumed, given the crude nature of the instrumentation, to be broadly similar. The implications of these data are that the oligosaccharide, unlike the polysaccharides employed in the earlier work, has caused little serious interference with the degree of protein-protein bonding, even at the inclusion level of 10 per cent. This view is confirmed, to some extent, by the figures for viscosity. Thus, if it is assumed that value for the control is dependent entirely on the resistance supplied by small pieces of coagulum, then clearly the inclusion of inulin (5 per cent) has caused the gel to disintegrate into smaller

Plate 1. *The typical structure of natural yogurt fortified with skimmed-milk powder; the bonding between the groups of casein micelles is evident, as are the whey-filled spaces[9]. (Source: A.Y. Tamime and M. Kalab)*

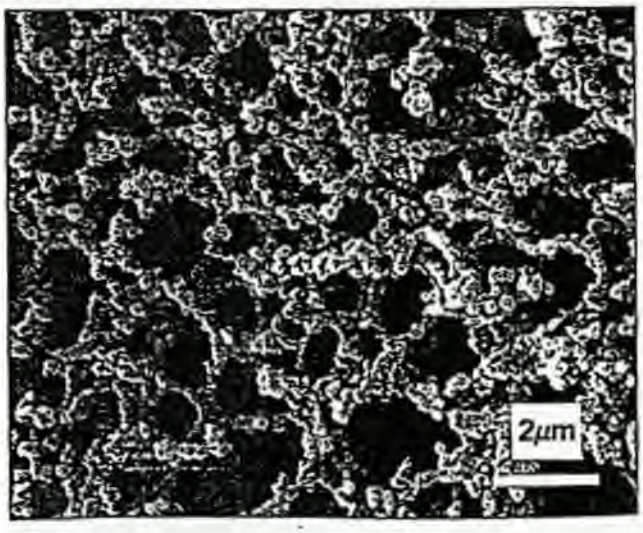

Table II. *Depth of penetration over a period of ten seconds of a standard cone and spindle into set yogurts of 16 per cent total solids and levels of inulin indicated; viscosity measures as described in text*

Level of inulin (%)	Depth of penetration (mm)	Viscosity (centipoises × 10^6)
Nil	22.0	3.79
1	23.0	3.25
5	20.0	3.04
10	20.8	3.62

Note: All readings at 5°C and the means of four samples

fragments that cause less "drag" on the spindle, i.e. there ould appear to have been some, albeit limited, interference with the network of protein-protein bonds. At the 10 per cent inclusion rate, it would reasonable to anticipate that both the size of the gel fragments and the viscosity would be reduced even further. However, it would appear that any loss in viscosity resulting from fragmentation of the protein has been almost nullified by the improved viscosity of the whey phase. This latter observation suggests that the overall impact of the inulin has been small and that, in practice, it could be incorporated into yogurt at a level determined by dietary considerations alone. This conclusion was further supported by the facts that:

- the rate of acid production by the starter culture was identical in all the milks; and
- a taste panel was unable to distinquish, on the basis of flavour, between the three batches of yogurt that included inulin; in a Triangular Test[12], the slightly improved "mouth feel" of the yogurt with 10 per cent inulin made it distinguishable from the other samples.

Conclusions

The cost of pure inulin as extracted from dahlias, chicory or Jerusalem artichokes might well, at least at present, preclude its use in yogurt on an industrial scale. However, there appears to be no reason why artichokes or chicory could not be grown as a source of inulin, just as potatoes are exploited for their starch[1,13,14], so rendering a commercial grade of the oligosaccharide available to the dairy industry.

Obviously, bio-yogurts are currently able to offer a bifidogenic dimension to the diet of interested consumers, but an alternative route for stimulating the native colonic flora would appear to merit serious investigation, particularly as the organoleptic quality of natural yogurt containing inulin, as well as a fruit-flavoured derivative, was excellent.

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YOGURT— Perceived Regulatory Trends in Europe

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Yogurt — Perceived Regulatory Trends in Europe**

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MANY consumers have a concept of yogurt as a rather pleasant, custard-like product, sharp to the taste and coming in a variety of fruit flavours. The more discerning may be aware that its manufacture involves a bacterial fermentation, and yet few concern themselves as to exactly what yogurt is or should be like. Yet these are exactly the questions which are now arising as the drive for national and/or international standardisation gains momentum. It is the purpose of this paper, therefore, to examine some of the standards that have been proposed for yogurt, and to assess what may or may not be achieved if current regulatory trends become hardened into reality.

Chemical Composition

As far as Europe is concerned, yogurt is based on cow's milk, and the available standards imply that the nutritional value of yogurt shall not be less than the milk from which it has been derived. The exception to this position is fat, and, as shown in Table 1, yogurt, including usually the base of fruit yogurt, is precisely typed according to fat content.

The minimum SNF value has few critics, and similarly there are reasonable commercial, and possibly health, arguments for the introduction of the low fat yogurts. Attainment of, and determination of, these levels presents few problems (Davis, 1970, Davis and MacLachlan, 1974), but whether there is any need for the precise designation of all the types is a moot point. Similarly the question of labelling has to be finally resolved, for while "skimmed-milk yogurt" is rather unattractive, the phrase "low-fat yogurt" has been criticised as having a 'low calorie overtone' which is not justified by many fruit yogurts. Whether the Swedish system of a 'normal' and 'light' yogurt would be suitable compromise remains to be seen, but, for the present, the three (or four including 'Balkan' yogurt) category approach appears to be gaining ground.

The other aspect of chemical composition that merits attention is the introduction of additives of non-dairy origin, that is fruit, flavouring and colouring materials, and stabilisers. Some aspects of the current position in this area are outlined in Table II.

The trends revealed by this data deserve more than a casual comment, and for a number of reasons. Thus, if it is accepted that the proposed additives have received toxicological clearance, then their exclusion (total or partial) is presumably based on a desire to endow present-day yogurt with the characteristics of its Bulgarian ancestor. Whether this sentiment should be enshrined in detailed legislation is another matter, as is the question of whether precise regulations for the addition of stabilisers is really necessary. If the proposed U.K. standards can serve as an example, it is clear that these are based on current practice, but equally clearly, the increasing cost of the hydrocolloidal materials and the limitations of existing plant, will both serve to ensure that the employment of plant gums is kept to a minimum anyway. The validity of spelling out maximum permitted levels is, therefore, open to comment, as also is the validity of the proposed specification.

The reason for raising this latter point is that plant materials, such as guar gum or gum tragacanth, can be placed under the heading of 'dietary fibre' (Jenkins *et al*, 1976(a)). The extensive literature on fibre need not be considered here, but the value of the materials in stimulating intestinal peristalsis and adsorbing potentially toxic chemicals has found widespread acceptance. However, recently, the possible value of plant gums has received the attention of the physiologists, and it has been shown that guar gum can act not only as an effective hypocholesterolaemic agent, but can also significantly decrease post-prandial hyperglycaemia in diabetics (see Fig. 1.). The suspected correlation between chloresterol levels in the blood and coronary disorders makes the first finding of some interest to most European males, while the more easily controlled response of the blood sugar levels may also be no bad thing.

Against this background, the proposed specification looks rather different, with the unnecessary and/or undesirable starch being allowed at the 1% level, while the seemingly valuable unabsorbable carbohydrates are regarded with less favour. Obviously this proposal may simply reflect the status quo, but it would appear that an equally valid argument could be made out for a total

Table 1. Suggested standards for the chemical composition of yogurt in terms of milk fat and solids-not-fat (SNF)

Country of Origin	Types of yoghurt based on % fat			% SNF
	Normal	Medium	Low	
Czechoslovakia	3.5	—	—	—
Denmark	3.8	1.8-1.5	0.3	—
Finland	2.5	—	—	—
France	3.0	—	1.0	—
Italy	3.0	—	1.0	—
Netherlands	3.2	—	0.3	—
Switzerland	3.5	2.0	0.5	—
Sweden	3.0	—	0.5	—
United Kingdom	3.5	2.0-1.0	0.3	8.5
West Germany	3.5	1.8-1.5	0.3	—
FAO/WHO	3.0	3.6-0.5	0.5	8.2

After — Robinson and Tamime (1976).

Table II. Suggested standards for the chemical composition of fruit/flavoured yogurt in terms of non-dairy additives

Country Origin	Stabiliser	Fruit	Colour/ Flavour	Preservative
Denmark	Nil	10-15%	Nil	Nil
France	—	up to 30%*	As regulations	—
Italy	—	up to 30%*	—	In fruit SO_2-60ppm
United Kingdom	Starch Pectin 1-% Gelatin alginates agar Edible gums celluloses	up to 30%* 0.5%	As regulations	benzoic acid-120ppm

*Total additions.

After: Italian Standards (1972); French Standards (1974); UK Food Standards Committee (1975); IDF/Denmark (1975).

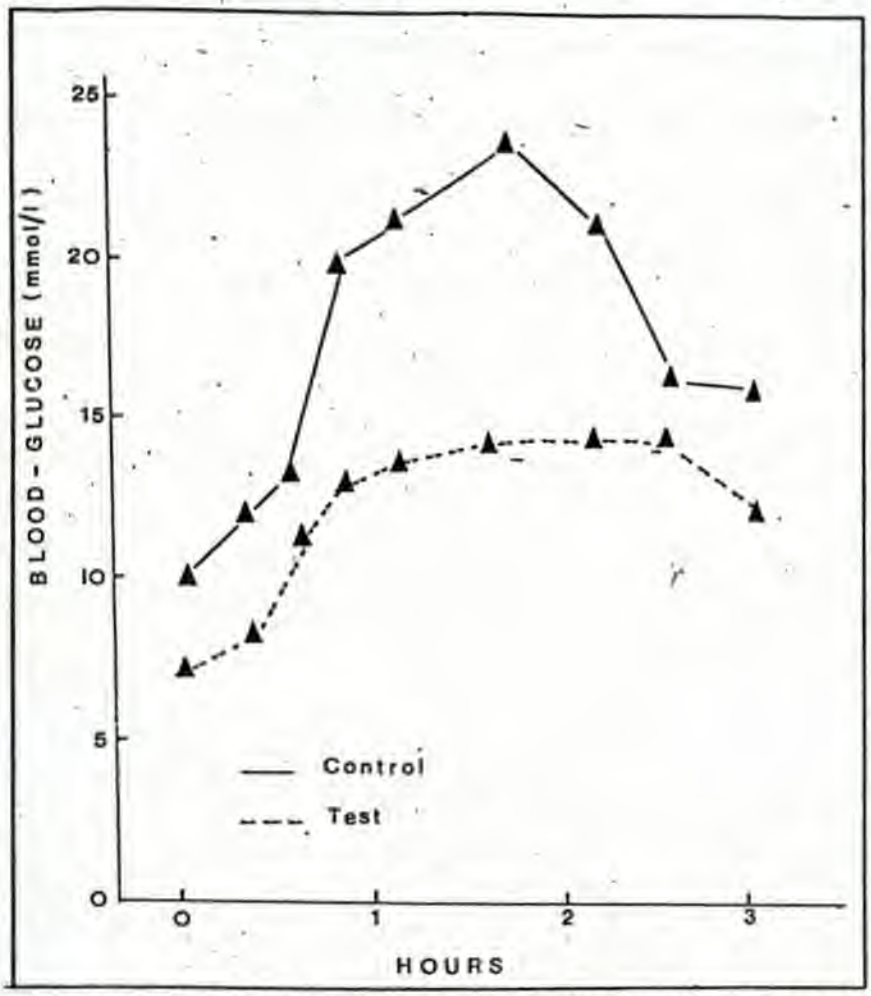

Figure 1. Individual blood-glucose levels (mmol/l.) of an insulin-dependent diabetic after taking a test meal with added 'dietary fibre'.

(After — Jenkins *et al*, 1976(b)).

ban on the use of starch as a stabiliser, with the gums permissible up to any level designated as necessary by the manufacturer. Such a proposal might prove unpopular, but it does raise the question as to just how far regulations are advisable merely to legalise an existing situation.

Microbiological and nutritional aspects

There are few people who would doubt that yogurt is a nutritious product, and, as shown in Table III, analyses bear this out. However, figures cannot tell the whole story, and the feeling persists that yogurt is something more than a collection of chemicals. The reported acceptance of yoghurt by people with demonstrated lactose-intolerance, is just one example that chemical analysis can only reveal a part of the story. Legislation cannot, apart from controlling some of the more optimistic advertisements, deal with nutritional value *per se*, but, in relation to the microbiology of yogurt, the arguments are somewhat intermixed.

For production purposes, it is stipulated that the requisite lactic acid must arise through the action, mainly, of *Lactobacillus bulgaricus* inconjunction with *Streptococcus thermophilus* or a similar lactic acid bacterium.

However, the need for yogurt to possess a specific identity, and the established symbiotic relationship between certain strains of *L. bulgaricus* and *Str. thermophilus*, would suggest that this more restrictive requirement should apply. Certainly France and Holland appear to be taking this line, and there would probably be few objections if other European countries followed their example.

Controversy does arise, however, once processing has finished, and while Germany and the United Kingdom seem agreed on the concept of 'pasteurised yogurt', other National Committees are insisting that yogurt must contain "abundant and viable bacteria"; indeed some countries, such as Holland, are considering the advisability of specifying 'minimum numbers' for bacteria in the retail product. This approach has also been advocated by Davis *et al* (1971), and it is probable that most samples could conform to the type of standards shown in Table IV. However, the data also indicates that retail samples are on the market with seemingly low counts of lactobacilli. Assuming that these figures are representative, then the question of 'minimum numbers' becomes more critical, particularly as 'advisory recommendations' could end-up as 'legal requirements' within the EEC context.

Two aspects would appear to merit attention, namely does the consumer expect an abundance of viable bacteria in yogurt, and does the presence of the bacteria contribute to the quality of the product? Obviously opinions differ considerably over the first question, and there appears to be no objective data from Europe on which to comment. However, trends in the United States could be relevant, and it has been suggested (Kroger and Fram, 1975) that the 'new generation' of consumers is unaware that yogurt contains live bacteria, and is not especially concerned anyway. This attitude does not argue in favour of pasteurisation, but it does suggest that concern over 'numbers' may be rather misplaced. The issue of quality is also difficult to quantify, but the general impression is that the flavour of pasteurised yogurt is someway behind its normal counterpart. This view implies that consumer reaction alone will inhibit any appreciable change to 'heat-treatment', for while brand loyalty may not be appreciable in respect of yogurt, brand rejection is an entirely different matter. The situation is aggravated further by the absence of any standard method of enumeration for the yogurt organisms, and there might be some justification in suggesting that, apart from ensuring accurate labelling and hygenic quality, attempts at pursuing microbiological conformity should be avoided.

Quality Appraisal

The hygienic quality of yogurt should present few problems, but, in general, organoleptic considerations have tended to be ignored. The monitoring of acidity has been proposed in Holland (Netherlands Standards, 1967), while in France (French Standards, 1974) a compositional figure for lactic acid is included in the Regulations. However,

Table III. Some nutrients in yogurt and milk; all figures per 100g.

Material	Protein	Fat (g)	Carbo-hydrate		Vit. C. (mg)	Riboflavin
Natural Yoghurt	5.0	1.0	6.4	180	0.4	0.26
Fruit Yoghurt	4.8	1.0	18.2	160	1.1	0.23
Milk	3.4	3.7	4.8	120	2.0	0.15

After: U.K. Food Standards Committee (1975).

outside these countries numerical values for acidity are not recorded, and, with 90% of the market occupied by fruit yogurts, the question of a 'traditional yogurt flavour' has become a minor interest. The relevance of consistency has not diminished, however, and casual observation would suggest that this area represents a major source of consumer complaint. Various quantitative techniques are available to assess the viscosity of yogurt, and, in technical terms, it would seem that the establishment of limits for a particular type of yogurt would be perfectly feasible. Whether such a proposal would go too far in removing brand distinctions is open to debate, but a trend towards more uniformity would have its adherents.

This tendency to consider the consumer more closely is reflected in the expanding role of organoleptic appraisal in the quality control of yogurt. One typical scheme is shown below (Pearce and Heap, 1974):—

Character Assessed	*Score*
Appearance and colour	5
Body and Texture	5
Flavour	5 x 2
	Total 20

Schemes, based on this format, are in regular use by the Government Control bodies of Denmark, Norway and Sweden. Whether regulations governing this form of appraisal will be formalised remains to be seen, but the quantitative nature of the rating scheme would suggest that regulatory standards could be introduced.

Discussion

In attempting to assess the drift in regulations concerning yogurt, no attempt has been made to distinguish between proposed legal standards and advisory recommendations, because, in the long term, both could become mandatory for the producer. Assuming that this view is valid, then the interested parties, namely the producers and the consumers, should perhaps take a hard look at the emerging situation. In particular it might be asked whether precise legal standards are necessary for a commodity like yogurt, and, if not, is there a workable alternative?

These questions imply no criticism of the various committees currently discussing the legislative position, but are concerned simply with raising the issue of whether legislation, perhaps advisable for international trade, is really required at national level. The relevance of this issue has been highlighted recently by the realisation that legislation can emerge in a form that apparently benefits no-one. The recently proposed ban on the sale of fresh poultry is just one example of the type of legislation that, despite being of no interest nationally, could easily have passed onto the Statute Book. The eradication of apples below 50 mm. from the domestic retail markets, is another example of a seemingly arbitrary ruling that helps neither the consumer nor the producer. If the characteristics of yogurt become similarly inflexible, perhaps under the auspices of the EEC, it may prove, in the long term, to be to the disadvantage of all the interested parties.

It is assumed premise of all regulations that they are necessary, but if, in the case of yogurt, this conclusion were rejected, then the question arises as to whether the absence of legislation would really matter. Obviously opinions will differ, but a good case can be put forward to suggest that little advantage will accrue from the introduction of precise specifications. Thus, there is already a measure of agreement between manufacturers as to what shall be labelled as 'yogurt' and a minimum of additional discussion could remove any undesirable anomalies. Market research could be employed to elicit the consumer viewpoint, and the end-result would be an agreed, general characterisation of yogurt that would meet the interests of both the manufacturers and the consumers. Adherence to the spirit of this agreement would be the responsibility of the producers and/or an appropriate federation, and there is every reason to believe that such self-imposed strictures would hold. Thus, the growth potential of yogurt makes it vital for all producers to ensure that an attractive product image is preserved, while, at the same time, a manufacturer who does attempt to 'cut corners' will soon find his customers looking to other brands.

It is this built-in system of consumer protection that makes legislation or even advisory regulations somewhat redundant, so leaving the way clear for the dairy industry to be self-regulating. Aspects of advertising and hygiene are already covered by existing Acts, and it may be that the introduction of further standards should be resisted. Certainly this attitude can be found outside Europe, and it would seem that both Australia and New Zealand presently restrict Government involvement, in respect of yogurt, to matters of public health, while Canada has, as a declared policy, no intention of introducing compositional standards. In this latter instance, it is felt that free market competition together with the responsible attitude of producers is ample to ensure the maintenance of quality. If such a system works in the Canadian situation, then why not elsewhere, and it is, perhaps, time to decide that, at the National level at least, specific regulations of the type that were discussed earlier are both unnecessary and irrelevant to a modern European society.

Table IV: Range of counts of yogurt organisms in selected retail products

	Natural	Strawberry	Blackcurrant
Str. thermophilus x 10^6/ml.	10 — 820	35 — 1100	80 — 1850*
	—	200	—
	—	189	—
	—	54	—
	—	250	—
L. bulgaricus x 10^6/ml.	11 — 680	5 — 360	5 — 400*
	—	6	—
	—	80	—
	—	1	—
	—	150	—
	—	23	—
Suggested standard	V 10		

*After: Davis and McLachlan, 1974.

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REVIEW ARTICLE

Fermented milks and their future trends. Part II. Technological aspects

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INTRODUCTION AND HISTORICAL BACKGROUND

Even today the microbiological quality of raw milk is often very poor, and centuries ago the situation must have been infinitely worse. It is not surprising, therefore, that many communities acquired a taste for 'sour milk' or that, with time, techniques were developed to ensure that the process of souring followed a particular pattern. The constant use of the same vessels, or the addition of fresh milk to an on-going fermentation, would have led to the gradual evolution of locally popular products.

The nature of these products would have differed from region to region depending on the indigenous microflora, which in turn reflected the climatic conditions of the area. In Scandinavia for example, mesophilic species might be expected to dominate, and hence a taste for milks fermented by *Lactococcus* and *Leuconostoc* spp. might be

expected to prevail (Marshall, 1984). In the Middle East, the thermophilic species would have led to yogurt-like products, while in the Caucasian mountains a range of beverages derived from a mixed culture of yeasts and lactic acid bacteria found popularity (Kemp, 1984).

Many of these geographical associations have long since broken down, so that yogurt, to take one example, now enjoys world-wide consumption. Nevertheless, the species composition of modern starter cultures largely mirrors microfloras associated historically with the products concerned, and this characteristic provides, therefore, an appropriate means of categorizing the various milks.

The microbiological aspects of fermented milks, including the latest nomenclature of the organisms employed, have been discussed in Part I of this review (Marshall, 1987) and in Part II the aim is to consider the recent technological developments that have taken place.

DEFINITION AND CLASSIFICATION

The International Dairy Federation (IDF) definition of fermented milks (IDF, 1969) takes into account the raw material, i.e. milk from cow, goat, sheep or buffalo; processing conditions; the fermentation process achieved using specific micro-organisms.

These general categories of fermented milks cover the main criteria of manufacture of these products. The exact names given to the fermented milk products which are manufactured throughout the world are not well established, but it is probable that there are several hundred. A monograph on fermented milks is being prepared by IDF Group B35.

In view of the fact that cows' milk is widely produced in different parts of the world, it is possible to suggest that the term fermented milks could be reserved for those products which are manufactured from cows' milk, and that the names of other cultured dairy products (from milk of different species of mammal) should imply the type of milk used. For example, the term yogurt should be reserved for the product made from cows' milk, dahi from buffaloes' milk, and similarly for other yogurt varieties produced from different milks.

For convenience, however, it was decided in the present review that a scheme for the classification of fermented milks which would be acceptable to both scientists and manufacturers should be based mainly on the method of fermentation. A generalized scheme for the classification of fermented milks, including some examples, is given in Table 1.

PATTERNS OF PRODUCTION AND CONSUMPTION

There are no exact data for the world production of fermented milks. However, the data in Fig. 1 illustrate the pattern of consumption of yogurt and buttermilk (in some instances other fermented milk products are included) in some of the IDF member countries. It is evident that Finland and the Scandinavian countries have the highest *per capita* consumption of fermented milks in the world, followed by most countries in Western Europe, Iceland, India and Israel. Thus, if the pattern of consumption in these countries (see Fig. 1) is considered to be equivalent to the total production, then in 1984 the production of fermented milks was in the region of 9·5 million tonnes (IDF, 1986). In 1981, the total production of acidophilus milk and other dairy foods containing *Lactobacillus acidophilus* in Denmark, Brazil and USSR was 2, 21 and 198 thousand tonnes respectively (IDF, 1983).

Table 1. *Classification and some examples of fermented milk products*

Type of fermentation	Traditional name	Country of origin	Microflora present
I. Lactic acid			
A. Mesophilic	Taetmjolk, Filmjolk, Lattfil, Langfil	Scandinavia	*Lactococcus lactis* subsp. *lactis*. biovar *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*
	Maziwa lala	Kenya	*Lactococcus* spp.. *Leuconostoc* spp.
	Ymer	Denmark	*Lact. lactis* subsp. *cremoris*, biovar *diacetylactis*
B. Thermophilic	Yogurt	Most countries	*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus*
	Bulgarian buttermilk	Bulgaria	*L. delbrueckii* subsp. *bulgaricus*
	Yakult	Japan	*L. casei*
	Liquid yogurt	Korea	*L. delbrueckii* subsp. *bulgaricus* or *casei* or *helveticus*
C. Therapeutic	ACO-yogurt	Switzerland	Yogurt culture, *L. acidophilus*
	A-38 fermented milk	Denmark	*L. acidophilus*, mesophilic lactic acid bacteria
	Acidophilus milk	Most countries	*L. acidophilus*
	AB-fermented milk	Denmark	*L. acidophilus*. *Bifidobacterium bifidum*
	AB-yogurt	Denmark	As above plus yogurt culture
	Biogarde‡	Federal Germany	*Str. salivarius* subsp. *thermophilus*. *L. acidophilus*. *Bf. bifidum*
	Bioghurt‡	Federal Germany	*Str. salivarius* subsp. *thermophilus*, *L. acidophilus*
	Bifighurt‡	Federal Germany	*Bf. bifidum*
	Mil-Mil E, Miru-Miru, Yakult	Japan	Yogurt culture, *Bf. bifidum*; *L. acidophilus*. *casei*. *Bf. breve*; *L. casei*
II. Yeast-lactic acid			
	Kefir	Russia	Refer to Part I of this review article*
	Koumiss	Russia	Refer to Part I of this review article*
	Acidophilus-yeast milk	Russia	*L. acidophilus*, lactose-fermenting yeasts
III. Mould-lactic acid			
	Viili	Finland	Similar to filmjolk, *Geotrichum candidum*
IV. Miscellaneous fermentation			Refer to Part I of this review article*

* Marshall (1987).

The above production figures do not include countries in the Middle East and North Africa where the consumption of fermented milks is high, and hence the world production figures for such products should be estimated to be in excess of 9·5 million tonnes.

Kurmann (1984) analysed the pattern of consumption of fermented milks in the member countries of the IDF, and noted that the factors which influence production and consumption include cattle breeding and milk production: countries with high milk production in the northern *vis-à-vis* southern hemisphere tend to have developed the habit of consuming more milk, and there is greater impetus to manufacture fermented milk products from the surplus milk. The type of diet also had an effect: consumption of fermented milks is greater among communities in

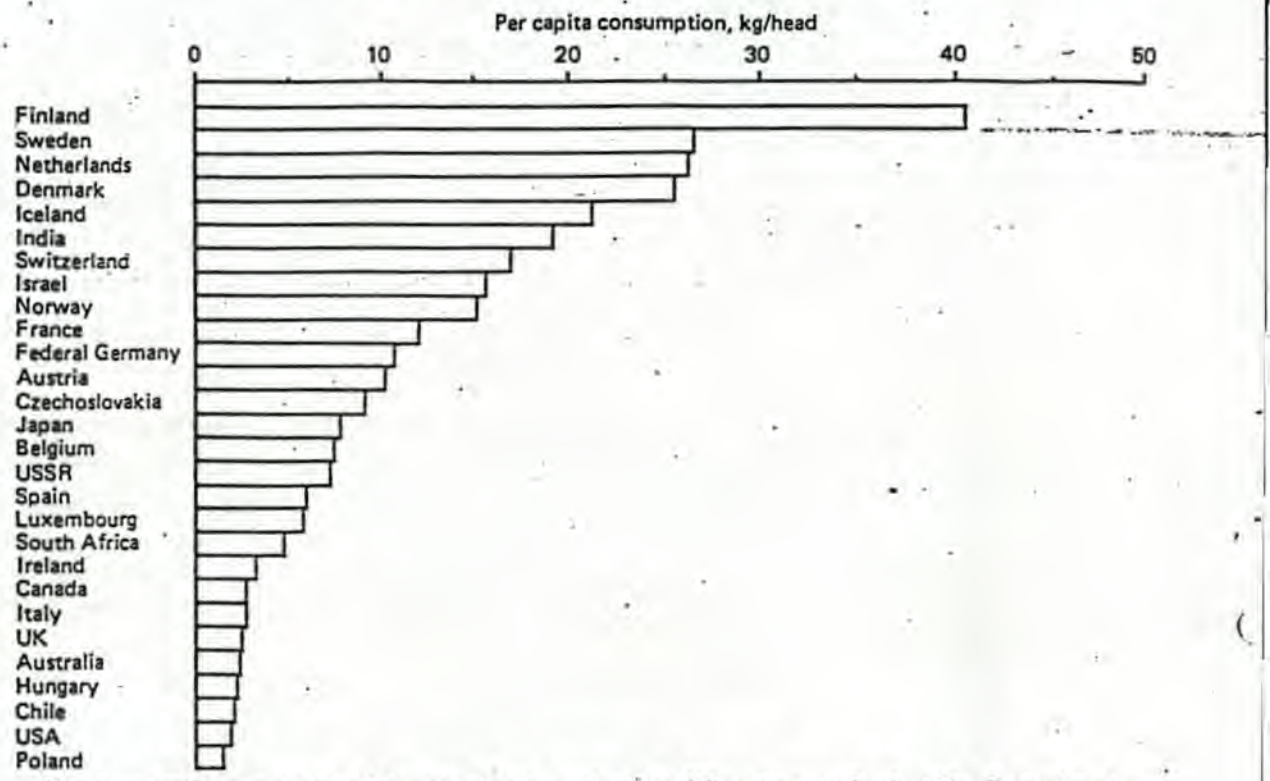


Fig. 1. Annual consumption of fermented milk products in different countries in 1984. Reproduced from International Dairy Federation (1986).

different regions of the world who have adopted mixed diets, as compared with the exclusively vegetarian (South America, Africa and the Far East where the consumption of fermented milks is very low) or animal diets which are popular among nomadic tribes. Although the latter type of diet may include lactic-type foods, the level of consumption is low, e.g. inhabitants of the polar region and Asiatic part of Russia. A decrease in milk and butter consumption among the industrialized nations over the past two decades has been paralleled by an increase in the consumption of fermented milks, fresh cheeses and various liquid dairy products. Other miscellaneous factors that contribute to the trend of increasing consumption of fermented milks may include level of income, availability of an organized distribution system and diversification in the type of fermented milks sold to the consumer.

Finally, it is of interest that none of the great religions of the world forbids the consumption of fermented milks.

TECHNOLOGY OF MANUFACTURE

Over the past three decades, a wide range of research papers, reviews and text books has been published on the manufacture of fermented milks. It is beyond the scope of this publication to review the literature in detail; the following are recommended for further reading: in English (Eller, 1971; Rašić & Kurmann, 1978, 1983; De, 1980; Kosikowski, 1977; Tamime & Robinson, 1985), in Russian (Rostrosa, 1973; Koroleva, 1975), in Bulgarian (Nakashev & Petrova, 1977; Chomakov, 1978), in German (Klupsch, 1984) and in Arabic (Abou-Donia, 1975).

Specifically tailored processes have been developed for the manufacture of the different types of fermented milks, but in practical terms most schedules of

manufacture have much in common. For this reason, the technical aspects of production of the different types of fermented milk will be considered under the one heading, i.e. yogurt, but details of temperatures and duration of incubation merit individual consideration; the plant and equipment are almost universal.

Lactic fermentations

This type of fermented milk is the most popular product in the dairy industry. The starter cultures employed are mainly lactic acid bacteria, and some examples are given in Table 1. Most of these products are made using a mixed starter culture (yogurt, ymer, or buttermilk), but in some instances a single organism is employed (Bulgarian buttermilk, acidophilus milk and yakult).

Mesophilic

The term mesophilic fermented milks is used for those products employing microorganisms whose growth optima range between 20 and 30 °C; the well-defined microflora consists mainly of *Lactococcus* and *Leuconostoc* spp.

Cultured buttermilk

A pasteurized fermented skim milk, mainly made in USA and Ireland, and traditionally the by-product of buttermaking after churning ripened cream. The commercial starters may consist of *Lact. lactis* subsp. *lactis*, subsp. *cremoris*, biovar *diacetylactis* and *Leuc. mesenteroides* subsp. *cremoris*. The leuconostoc is a flavour-producing starter able to synthesize diacetyl from citrate with CO_2 as a by-product. When the citrate level is depleted, the diacetyl is catabolized to acetoin, and addition of 0·05–0·10 % Na citrate to the milk has been recommended by Vedamuthu (1978, 1985) in order to prevent the conversion phase. Loss of diacetyl concentration in buttermilk can occur if the fermentation exceeds the level of 0·78 % lactic acid (Vedamuthu, 1985). A green flavour in buttermilk is an undesirable characteristic, and is due to an excessive accumulation of acetaldehyde by the *Lactococcus* spp.; inclusion of *Leuc. mesenteroides* subsp. *cremoris* helps to catabolize it (Marshall, 1984).

Buttermilk

A fermented skim milk or low fat whole milk (1·7 % fat) which is preheated and homogenized (first stage 13·8 MPa, second stage 3·5 MPa) before pasteurization. The time/temperature employed may vary from 85 °C for 30 min to 95 °C for up to 5 min. The milk is cooled to 22 °C, inoculated with 1 % starter culture (*Lact. lactis* subsp. *lactis* and *Leuc. mesenteroides* subsp. *cremoris*), and at pH 4·6 the coagulum is cooled to 5 °C and packaged (Kosikowski, 1984; Vedamuthu, 1985). The consistency of the buttermilk is improved by fortification of the skim milk with 1·5–2·0 % skim milk powder, the addition of stabilizer (0·01–0·02 %), and/or the use of specially selected strains of lactococci that produce capsular slime.

Traditionally, buttermilk used to contain butterflakes, and this can be achieved by the addition of lyophilized butterflakes at a rate of 0·02 % to buttermilk before packaging.

Fruit flavouring has not been a successful commercial process in the United States owing to colour changes in the presence of the lactic starter culture, but the addition of 0·02–0·2 % citric, malic or phosphoric acids can prevent this defect (Kosikowski, 1984). It is also recommended that the addition of 0·1–0·2 % Na citrate helps the starter bacteria to produce diacetyl rather than acetaldehyde or acetoin.

However, Lundstedt & Corbin (1983) suggested that the fermentation of buttermilk could be controlled by cooling the product at pH 5·2 to stop the metabolic activity of *Lact. lactis* biovar *diacetylactis*, or by direct acidification of the product to pH 4·5 with lactic acid. The latter approach could not be considered as a biosynthetic process, because most of the acid is added, rather than secreted by microorganisms.

The microbiological quality of raw milk, especially with regard to *Pseudomonas* spp., can affect the flavour of buttermilk, and a level above $50–100 \times 10^3$ cfu ml^{-1} can cause bitter, unclean and medicinal flavours (White, 1978). These organisms produce heat-resistant enzymes able to reduce diacetyl and produce acetylmethylcarbinol. A good flavoured buttermilk contains a diacetyl to acetaldehyde ratio of 4:1, and hence a reduction in diacetyl is not desirable (Frank, 1984).

The decline in buttermilk consumption in the USA has been off-set by a demand for the product in the bakery industry, and a long holding time during the heating of the milk (85 °C for 30 min) can achieve the 80% denaturation of the whey proteins needed to obtain the 'rise' in baked goods (Vedamuthu, 1985).

Ymer

A Danish cultured milk product containing 3·5% fat, and 11% solids-not-fat (SNF) including 5–6% protein. The starter culture consists of *Leuc. mesenteroides* subsp. *cremoris* and *Lact. lactis* biovar *diacetylactis* (Anon. 1969).

Traditionally, the fermented skim milk (pH 4·6) is cut and warmed indirectly. This allows syneresis to occur, and 50% of the original volume is removed as whey; fat is then blended with the product, which is homogenized, cooled and packaged. This method of production results in a 50% loss of the whey proteins and calcium. At present, an ultrafiltration (UF) process is used to produce ymer commercially in Denmark, and according to Samuelsson & Ulrich (1982), the manufacture stages are as follows: warm the milk to 55 °C and separate the cream; pasteurize the skim milk at 92 °C for 15 s; cool to 55 °C and concentrate the milk by UF; standardize with cream to 3·5% and homogenize (19·6 MPa) at 65 °C; heat the milk to 85 °C for 5 min, cool to 20–22 °C, add culture and incubate for 20 h; stir coagulum, cool to 5 °C, store for 24 h, stir, package and distribute.

Lactofil

A Swedish concentrated cultured milk product similar to ymer, the only evident differences being that the fat content is 5%, and the culture used is similar to that for buttermilk and filmjolk.

Filmjolk

A Scandinavian sour milk; the fat content ranges between 0·5 and 3% depending on the brand, and the starter cultures include the acid producers *Lact. lactis* subsp. *lactis* and subsp. *cremoris*, and the flavour and aroma producers *Lact. lactis* biovar *diacetylactis* and *Leuc. mesenteroides* subsp. *cremoris* (Anon., 1980; Spetsig, 1983).

Incorporation of air into the milk or the cultured product during production can give rise to the defects of whey separation, granulation, lumpiness and thin consistency; hence de-aeration of the milk is an important stage during processing. According to Anon. (1980), the filmjolk process line could be described as follows: heat whole milk to 78 °C, and transfer to de-aeration vessel; separate the milk, and use part of the cream for in-line standardization of the skim milk; homogenize at 10–20 MPa and at a temperature of 70 °C; heat the milk to 90–95 °C for 3 min, cool to 18–20 °C and inoculate with 1–2% starter culture; break the coagulum at

0·8–0·9 % lactic acid after 20 h; cool and package, taking care that the agitation system does not entrap any air.

Nordic ropy milks

Traditional dairy products in Norway, Sweden and other neighbouring countries where herbs are used to produce thick milk that is slimy and ropy. Traditionally, in addition to the mesophilic lactic cultures that are present naturally in milk and are of unknown composition, the leaves of *Pinguicula vulgaris* and *Drosera* spp. are added to milk, both plants being known locally as tatgras (thickening grass); thus, *tatmjolk* means ropy or thick milk. *Filbunk* is a sour, whole milk which is prepared to be consumed immediately. No doubt the role of the microflora is important, and studies carried out in 1982 on ropy milk made by the traditional process described by Linne (Alm & Larsson, 1983) resulted in a flavourful product with an apple-like aroma; a pH of 4·4, with a good consistency and no sign of syneresis; shelf-life up to 3 months at 8 °C; milk sugar reduced by one third, and folic acid content much higher than for other fermented milk products; the total viable count was $3{\cdot}5 \times 10^8$ cfu g^{-1}, and that of the aroma-producing bacteria was about $1{\cdot}1 \times 10^7$ cfu g^{-1}.

The increase of folic acid content in the Nordic ropy milk is mainly attributed to the starter culture activity rather than the presence of tatgras in the milk. Alm (1982) has studied the effect of fermentation on vitamin B content of milk and fermented milk products in Sweden, including ropy milk without the addition of tatgras. This last product was manufactured by Alm (1982) from cows' milk (3 % fat, w/v) which was heated to 90 °C for 30 min, cooled, inoculated with a mixed culture of *Lact. lactis* 'biovar *longi*' and *Leuc. mesenteroides* subsp. *cremoris* and incubated at 17–18 °C for 20–22 h (pH range 4·5–4·6). The reported increase in the folic acid content of this type of ropy milk was more than twofold after one day's fermentation. Similar increases in the level of folic acid content of other fermented milks has been reported by different researchers in many parts of the world (see Alm, 1982).

Thermophilic

Thermophilic fermented milks are those products which consist mainly of lactic acid bacteria with an optimum temperature range of 37–45 °C, and the defined microflora consist of *Lactobacillus* spp. and *Streptococcus* spp.

Yogurt

A fermented milk product that has been developed over thousands of years around the Mediterranean basin, the Middle East and India. The product(s) is known as *laban* or *leben* (in Lebanon and most neighbouring countries), *lben* (in Morocco), *dahi* (in India) and *zabadi* (in Egypt). Under rural conditions, it is safe to conclude that the starter culture is a mixed flora containing mesophilic and thermophilic lactic acid bacteria and lactose-fermenting yeasts (Marshall, 1987; Abou-Donia 1984; Benkerroum *et al.* 1984; Arrizza *et al.* 1983; Tantaoui-Elaraki *et al.* 1983). Yogurt, including fruit-flavoured yogurt, which has been developed over the past few decades in the developed countries, and has been extremely successful in finding universal acceptance, could be considered similar to laban, leben or zabadi. The only difference is that the starter culture is of a defined thermophilic microflora, and consists mainly of *Streptococcus salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.

Cows' milk is generally used for the manufacture of yogurt, and relevant data regarding yogurt made from goats', sheep's or buffaloes' milk have been well documented elsewhere (De, 1980; Kurmann, 1986; Kehagias *et al.* 1986, Abrahamsen, 1986; Abrahamsen & Holmen, 1981; Kehagias, 1986; Rysstad & Abrahamsen, 1987).

A generalized scheme for the manufacture of yogurt is illustrated in Fig. 2. The factors that can affect the quality of the product include:

(1) Preliminary handling of the milk. The reception and/or handling of the milk in a factory for the production of fermented milks is discussed elsewhere by Rašić & Kurmann (1978); Tamime & Robinson (1985).

(2) Standardization/fortification of the basic mix. The fat content in the milk varies in relation to the species of mammal and the stage of lactation, and is adjusted to suit market demands or to comply with existing legal standards. The methods employed for the standardization of the fat content in milk are removal of fat from full cream milk, mixing full cream milk with skim milk, addition of cream to skim milk and the use of standardizing centrifuges. Fortification of the milk SNF is synonymous with standardization of the SNF in the yogurt milk, i.e. lactose, ash and protein. The protein fraction affects the physical properties (mainly viscosity/consistency of the coagulum), and an account of the role of protein in yogurt has been published recently by Robinson & Tamime (1986*a*).

A survey carried out in Scotland between 1984 and 1985 (Tamime *et al.* 1987), showed that the chemical composition (%) of yogurt (natural set and/or stirred) varied in total solids (TS) (12·0–18·7), fat (0·6–1·5), protein (4·5–6·4), lactose and other sugars (6·2–10·0), and ash (1·0–1·4).

It is suggested that the desired SNF content in the yogurt could be achieved using addition of milk powders (e.g. skim, full cream, whey or caseinates), concentration (e.g. by evaporation (EV), UF or reverse osmosis (RO)), or addition of miscellaneous products such as soyabean or leaf protein.

The first two methods are widely used in the dairy industry, and the final choice is dictated by the availability of the raw materials, or the capital investment required for the installation of EV, UF or RO equipment. The general recommendation for the fortification of the yogurt milk with dairy powders is at a rate of 2–4%; above this level the yogurt has a distinctive powdery taste, and becomes more expensive to produce. In EV and RO, the basic mix is concentrated to around 14–15% TS, and for UF to 12% TS with a view to maintaining the protein content at 5%.

Physical properties of the coagulum are primarily dependent on the casein:non-casein protein ratio. The work of Modler & Kaláb (1983) and Modler *et al.* (1983) suggests a ratio of 2·89:1·0 in yogurt made from skim milk powder, milk protein concentrate or whey protein concentrated by UF; the gel strengths of the coagula were similar, as were the degrees of syneresis. However, Tamime *et al.* (1984) concluded that a ratio of 3·2–3·4:1·0 produced a good yogurt (using skim milk powder, EV, RO or UF), while a ratio of 4·62:1·0 in yogurt which was fortified with Na caseinate gave a rough, coarse texture. The protein content of these yogurts was maintained between 5·0 and 5·6%. In another study, the degree of syneresis decreased as the milk solids level was increased from 10 to 30%, owing to changes in size of the casein particles (large at 10% solids and small at 30% solids) which formed the chains/cluster of the protein matrix (Harwalkar & Kaláb, 1986).

Milk powders (e.g. full cream, skim, buttermilk, and whey) are easily reconstituted in the yogurt milk at refrigeration temperature, or at around 40 °C.

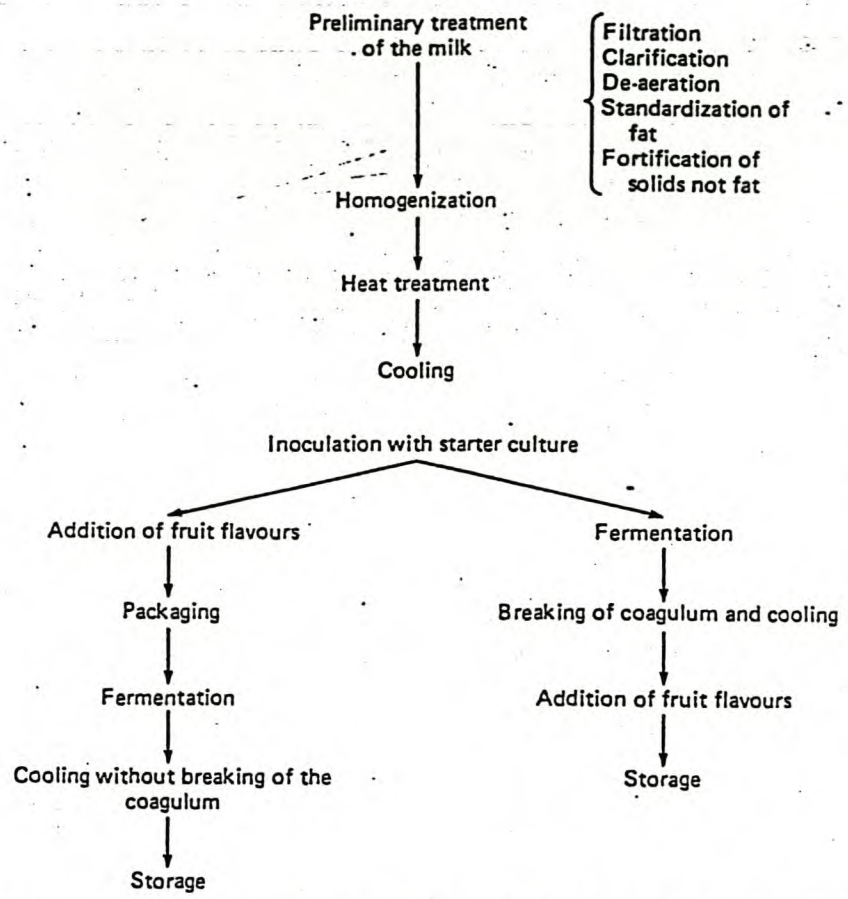


Fig. 2. Flow diagram illustrating the main stages involved during the manufacture of most fermented milks

The main concepts of recombination have been fully documented in previous IDF Bulletins (IDF, 1979, 1982; Bøjgaard, 1986). The wettability of caseinate powders may pose some problems, but rehydration at higher temperatures and the use of high speed mixers could minimize this difficulty. The alternative systems employed for the fortification of the yogurt milk by the concentration methods (EV, UF and RO) have been reviewed by Tamime & Robinson (1985).

Filtration of the reconstituted products is a recommended procedure for the removal of undissolved or scorched particles from the milk and to minimize the deposition of milk solids in the heat exchanger. In addition de-aeration of the basic mix is sometimes practised in order to provide the appropriate growth conditions for the starter cultures.

At this stage of processing, other dried ingredients are added to the yogurt milk, i.e. sugar and stabilizers. The former is added to sweeten, and the recommended level is governed by consumer acceptability, but levels greater than 10–12% can reduce the metabolic activity of the starter culture. Stabilizers, which are hydrophilic in nature, are added in order to improve the viscosity of the product and to prevent whey syneresis. Some examples of stabilizers are starch, gelatin, locust bean gum, guar, agar, alginates, pectin, carrageenan, or combinations of these compounds, and the recommended level of use is up to 0·3%. Higher levels can increase the consistency but the flavour of the yogurt would be affected adversely. A comprehensive list of permitted stabilizers for yogurt has been reported by the Food and Agriculture Organization (1984). In some instances, syrups and liquid stabilizers

(e.g. gelatin) are added to yogurt milk after the heat treatment stage, i.e. at 40–45 °C, so that the microbiological quality of these products is critical.

(3) Homogenization. In industrial practice, homogenization of yogurt milk is carried out at 9·8–19·6 MPa and between 50 and 70 °C. Normally the process takes place during the upward flow of heating of the basic mix. Homogenization is efficacious in that it prevents the tendency of the fat to rise to the surface in the incubation tanks (for stirred yogurt) and in the retail container (for set yogurt); it reduces syneresis due to protein–protein interaction, thus improving the water holding capacity of the coagulum; it reduces the tendency of the small fat globules to coalesce, because they are coated with a new membrane component which consists mainly of casein submicelles; it improves the light scattering of the product, so that the yogurt appears whiter in colour; and finally it ensures uniform mixing of any dry ingredients added to the milk.

Homogenized milk has a tendency to foam because of the increased level of phospholipids in the skim phase, and it is strongly recommended that the incubation tanks should be bottom filled.

(4) Heat treatment. The application of heat to yogurt milk is a universal practice, and the reported time/temperature treatments can be classified as 85 °C/30–45 min, 90–95 °C/5–10 min, 115 °C/30 s or 149 °C/3·3 s.

Heat-induced changes in milk constituents described by Tamime & Deeth (1980) and Tamime & Robinson (1985) include destruction of undesirable organisms in milk; production of compound(s) which stimulates *L. delbrueckii* subsp. *bulgaricus*; production of volatile compounds which may contribute to flavour; changes in the ionic status of minerals which help to decrease the coagulation time; and changes in the physicochemical nature of the nitrogenous components of milk.

The last factor is important and could be considered as one of the major changes in relation to the manufacture of yogurt. Some of the heat treatments of yogurt milk mentioned above, i.e. 85 °C for 30–45 min or 90–95 °C for 5–10 min. are sufficient to denature the whey proteins and induce interactions between the κ-casein, β-lactoglobulin and possibly α-lactalbumin. These interactions are governed by the milk constituents and the type of milk used. Ramos (1978) reported the changes in the nitrogenous fractions of cows', goats' and sheep's milks (see Table 2).

It could be argued that such changes might be applicable also to yogurt milks which have been fortified by the addition of dairy powders, or by EV, RO or UF. Scanning electron microscopy and transmission electron microscopy are modern techniques which have been employed to study the microstructure of yogurt, i.e. casein/whey protein interactions, the formation of chain matrices and the aggregates or clusters of the casein micelles (see recent review, Robinson & Tamime, 1986*a*). The production of a firm yogurt coagulum in heated milks is probably primarily the result of interactions between casein and the denatured whey proteins.

Heating of the milk increases the hydrophilic properties of the coagulum and the stability of the yogurt gel. Labropoulos *et al.* (1981, 1984) concluded that the physical properties of yogurt manufactured from milk heated to 82 °C for 30 min, compared with 149 °C for 3·3 s, were best, and that the latter treatment is suitable only for the production of drinking yogurt or yogurt with thin consistency or low curd firmness. Similar observations were reported by Parnell-Clunies *et al.* (1986*a*, *b*) and Parnell-Clunies & Kakuda (1986) for whole milk heated at 85 °C for 10–40 min (vat process), at 98 °C for 0·5–1·87 min (high temperature short time – HTST) and at 140 °C for 2–8 s (ultra high temperature – UHT) for the manufacture of yogurt. However, they concluded that: (*a*) yogurt firmness and viscosity was increased with

Table 2. *Changes (%) in the nitrogenous fractions of milk from different animal species after heating at 80 °C for 10 min*

Casein	cow	<	goat	<	sheep
Non-casein N	goat	<	cow	<	sheep
Soluble protein	goat	<	cow	<	sheep
β-Lactoglobulin	cow	<	sheep		
Non-protein N	goat	<	sheep		

After Ramos (1978).

Table 3. *Milk processing for the manufacture of yogurt*

Labropoulos *et al.* (1981, 1984)	Parnell-Clunies *et al.* (1986*a*, *b*)	Schmidt *et al.* (1985)
Raw whole milk	Raw whole milk	First day
↓	↓	Separate whole milk at 37 °C
Prewarming at 60 °C	Heat treatment (see text)	↓
↓	↓	Fortify skim milk with 3% skim milk powder
Homogenize	Pre cool to 50 °C	↓
↓	↓	Warm to 49 °C and homogenize at 17·24 MPa (first stage) and 6·9 MPa (second stage)
Heat treatment (see text)	Homogenize at (105 kg/cm^2) 10·3 MPa	↓
↓	↓	Cool to 4 °C and store for 14 h
Cooling to 43 °C, inoculate with 3% separate culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*	Cool to 45 °C and inoculate with 3% starter culture	
↓	↓	Second day
Incubate at 43 °C for 4–5 h	Package (200–700 ml)	Heat treatment (see text)
↓	↓	↓
Cool and store at 4 °C for 12–48 h	Incubate at 42–44 °C until pH 4·6 ± 0·05	Cool to 42 °C
	↓	↓
	Cool and store at 5 °C for 42 d	Package (300 ml) and inoculate 0·5% (v/v) with each of *Str. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*
		↓
		Incubate at 40 °C to pH 4·3
		↓
		Cool in ice bath
		↓
		Store for 1 week at 4 °C

the vat process *v.* HTST and UHT treatments, but the highest water holding capacity of the coagulum was observed with the HTST process followed by the UHT and vat treatments; (*b*) although structural changes of the yogurt coagulum are induced by heat, other parameters, i.e. hydrophobicity, denaturation of individual whey proteins rather than total denaturation and changes in disulphide and sulphydryl groups should not be overlooked; (*c*) yogurt made by the vat process exhibited syneresis and a grainy texture, and the application of UHT could be limited by the weak texture of the coagulum; (*d*) the HTST process, i.e. heating milk at 98 °C for 1·87 min, represents the best process for industrial production.

Schmidt *et al.* (1985) prepared yogurt as described in Table 3, and they concluded that the effects of heating on yogurt texture were that the conventional yogurt exhibited higher firmness, viscosity and more syneresis that UHT yogurt; improved firmness and consistency of UHT yogurt was achieved at the longer holding time; and heating the milk at 138 °C rather than 149 °C was not, as reported by Labropoulos *et al.* 1981, 1984), as destructive to the yogurt texture.

The above studies represent the latest investigations on the effect of milk

processing conditions on the physical properties of yogurt. The design of these experiments is summarized in Table 3. It can be observed that the physical properties of set yogurt only have been examined. In practice, the coagulum of stirred yogurt is broken mechanically before cooling and before packaging, but in all of these studies the coagulum of set yogurt was broken mechanically after refrigeration, which is contrary to the normal practice; hence, the physical properties of stirred yogurt, and in particular the thixotropic behaviour of the product, may not be similar to those reported by Labropoulos *et al.* (1981, 1984), Parnell-Clunies *et al.* (1986*a*, *b*) and Schmidt *et al.* (1985). The method adopted for the production of yogurt by the last authors could be inconvenient for industrial application, because the production time is extended by one day.

The types of process which can be employed for the heat treatment of the yogurt milk are classified as batch or continuous. In the former type, multi-purpose tanks are used, and the processing cycle involves heating and holding the milk at the desired temperature, cooling the milk to incubation temperature, and cooling of the coagulum.

The continuous process (e.g. plate, tubular or scraped surface heat exchangers) is most commonly used in the industry, and the milk can be heated to any desired temperature. The holding section of the yogurt processing plant is built as an external unit so that for a short holding time (3 s) an extended piping system from the heating section is used; for a medium holding time (up to 10 min) a special spiral tube/cell or tubes in zigzag arrangement is used; while for a long holding time (up to 45 min) water-jacketed or insulated tanks are used.

The above-mentioned heating/holding systems have been discussed by Tamime & Robinson (1985).

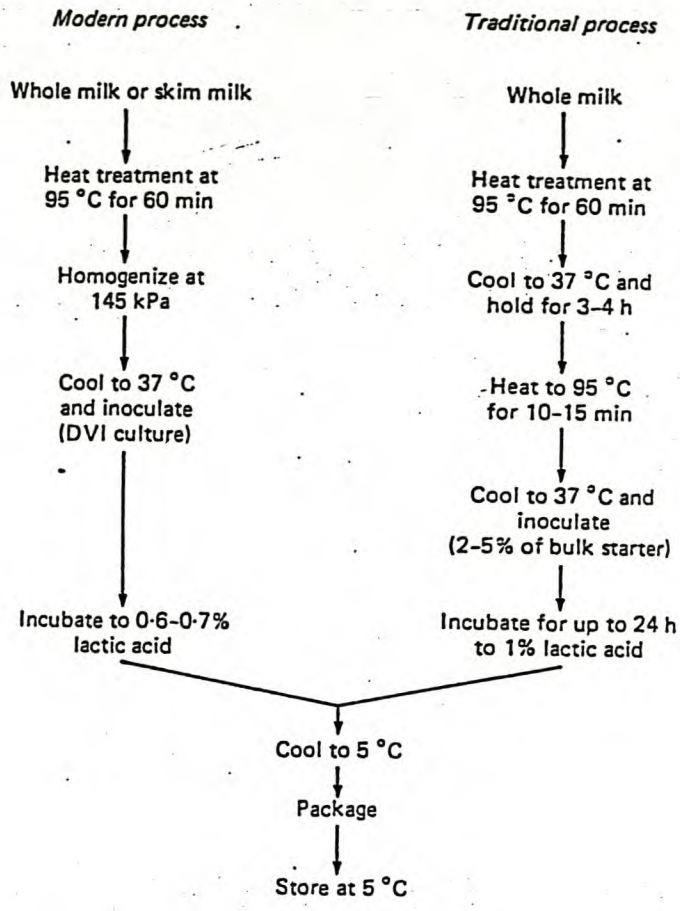
(5) Production. Methods of fermentation, cooling, addition of fruit flavours and packaging have not changed dramatically over the past decade. Illustrations (see Fig. 3) of these systems have been reported by Rašić & Kurmann (1978), Tamime & Greig (1979), Tamime & Robinson (1985) and Robinson & Tamime (1986*b*).

Yogurt-related products

Strained or concentrated yogurt and labneh products are popular in the Middle East and neighbouring countries. The rural method of manufacture consists of straining natural yogurt using a cloth bag, animal skin or earthenware vessel. The product is then known as *labneh* or *lebneh* (in Lebanon and most Arab countries), *tan* or *than* (in Armenia), *tulum* (in Turkey) and *leben zeer* (in Egypt).

Preliminary studies on the effect of using various strains of yogurt starter culture on the rate of whey drainage were first reported by Tamime & Robinson (1978), who concluded that strains producing hydrocolloidal polysaccharides are not suitable because of the longer time required for the removal of whey.

Until a few years ago, the factory scale production of labneh was similar to the traditional process, but this method is slow, cumbersome, and unhygienic and losses are high. Recently, the traditional practice has been up-dated for creamery scale operation, and new mechanical systems installed. With these, the yogurt coagulum is separated using a modified nozzle separator. Very low fat yogurt is used and cream is blended later with the concentrated product to the desired level. Dagher & Ali-Ghariebeh (1985) have produced labneh from heated yogurt by centrifugation for 5 min at different speeds, i.e. 4080, 7970 or 11700 *g* respectively. Organoleptically all these labnehs were similar to the control (cloth bag) samples. The same authors have used H_2O_2 and K sorbate to prolong the shelf life of the product, but these additives



After Chandan (1982), Robinson & Tamime (1981)
DVI, direct-to-vat-inoculation.

Fig. 3. Two alternative methods for the production of acidophilus milk

are not acceptable in certain countries. The production of labneh using a separator has been reported also by Salji *et al.* (1983) in Saudi Arabia. In another process (Kharrazi, 1984) for the production of labneh, yogurt is blended with brine (3–12% salt) and the mixture concentrated using a centrifugal separator.

UF methods have been used for the production of labneh, and two different systems have been reported. In the first the milk is concentrated by UF to the desired TS before heat treatment and fermentation (Abd El-Salam & El-Alamy, 1982; Veinoglou *et al.* 1978; Ibrahim, 1979), while the other system involves concentrating the warm yogurt by UF. The latter approach is similar to the production of quarg, and currently this method of processing is under review at the West of Scotland Agricultural College.

Although there are no exact data for the production of labneh in the Middle East, the reported annual production of this product in Saudi Arabia in 1982–3 was around 7 thousand tonnes (Salji *et al.* 1987*a*, *b*), and it is also produced in many other parts of the world including the United Kingdom. Table 4 shows the chemical composition of these products.

Chakka and shirkhand. This is Indian-type fermented milk and the former product is similar in composition to labneh (Table 4). The only evident difference is that chakka is manufactured from skimmed buffalo milk. Different starter cultures have been employed, i.e., mixed strains of lactic cultures (a) *Lact. lactis* subsp. *lactis*,

Table 4. *Chemical composition (%) of labneh and other related products*

Product	Total solids	Fat	Protein	Lactose*	Ash	Titratable acidity % l.a.	Reference
Labneh							
1. Lebanon							
Commercial	22·90	10·50	ND	ND	ND	1·66	Tamime (1977, 1978)
Commercial	21·78	8·40	ND	ND	ND	2·53	
Commercial	21·67	8·20	ND	ND	ND	2·08	
Standard	26·00	10·00	—	—	—		
2. Saudi Arabia							
Commercial†	24·61	8·14	10·43	4·91	1·07	1·05	Salji *et al.* (1983. 1987*a*,*b*)
Commercial‡	23·44	8·34	9·27	3·51	1·33	1·86	
Commercial§	20·54	6·42	9·02	2·86	1·14	1·91	
Standard	22·00	7·00	—	—	—	—	
3. United Kingdom							
Experimental	23·7	9·8	8·80	3·96	1·14	1·89	Tamime (1977, 1978)
Experimental	23·7	10·26	9·00	3·77	0·67	ND	
Commercial	22·95	11·04	5·81	5·10	1·00	1·62	Tamime (unpublished data)
Commercial	20·39	9·50	5·62	3·31	1·96	1·44	
4. Greece							
Experimental	ND	9·50	8·50	ND	ND	ND	Veinoglou *et al.* (1978)
Commercial	22·40	10·70	8·24	ND	ND	1·73	
Chakka							Patel & Abd El-Salam (1986)
India	23·00	trace	14	3·25	1·08	2·2	Patel & Chakraborty (1985)
Skyr							
Iceland							
Traditional	20·79	0·37	15·83	3·63	0·96	2·72	Tamime (unpublished data)
Commercial	17·49	0·22	12·65	3·85	0·77	1·86	
Labneh anbaris							
1. United Kingdom	31·18	4·78	18·60	6·95	1·25	ND	Tamime & Crawford (1984)
2. Israel	46·48	20·00	17·67	3·98	4·83	3·42	Rosenthal *et al.* (1980)

* In some instances % lactose was calculated by difference; † mean average of commercial plants in Central province; ‡ mean average of commercial plants in Western province; § mean average of commercial plants in remaining three provinces; ND not determined; — not specified.

biovar *diacetylactis* and *Leuc. mesenteroides* subsp. *cremoris* in a ratio of 1:1:1, (b) *Lact. lactis* subsp. *lactis* and *cremoris*, (c) *Str. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in a ratio of 1:1, and (d) *Lact. lactis* subsp. *lactis* and biovar *diacetylactis*. This last mixed culture, e.g. LF–40, has been accepted as the most suitable by many shirkhand manufacturers in India (Patel & Abd El-Salam, 1986). After fermentation the product is concentrated using a basket centrifuge at 900 *g* for 90 min. Shirkhand is produced by blending chakka with cream, sugar and cardamom, and its chemical composition (%) is as follows: TS 57–60, fat 5–6, protein 6·5–7·0, sucrose 40–43, reducing sugar 1·6–1·7, ash 0·49–0·55 and titratable acidity 1·05–1·10 (Patel & Abd El-Salam, 1986).

Experiments on the use of mutant strains of *Lact. lactis* biovar *diacetylactis* (parent S, and mutants S-95 and PM) for the manufacture of chakka have been reported by Khanna *et al.* (1982), which showed that the mutant strain PM was rated best by the panel of judges when grown in cows', buffaloes' or reconstituted milks.

Skyr. An Icelandic concentrated, fermented milk product which is manufactured from skim milk. The chemical composition of traditional and commercial skyr is illustrated in Table 4, and the protein content of the former product is around 16%.

Methods of production are similar to labneh (i.e. traditional using the cloth bag, and commercial using the nozzle separator).

The microflora of skyr consists of thermophilic lactic acid bacteria (*Str. salivarius* subsp. *thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. jugurti* and *L. helveticus*) and lactose-fermenting yeasts (Pétursson, 1949); during the fermentation of skim milk, some coagulant is added with starter culture. The fermented milk, before concentration, is called *petti*, which has the same origin as the Norwegian word, *taette*.

Post-fermentation heat treatment of skyr is widely practised in Iceland, and data regarding the effect of seasonal variation on the compositional quality of skyr are well documented by Ragnarsson *et al.* (1983) and Reykdal *et al.* (1985). Icelandic skyr is now flavoured with strawberry or bilberry, and in some instances cream is blended with concentrated product.

Labneh anbaris. A cheese-like product which has twice the level of milk solids of labneh and is preserved in olive oil. Similar products are produced in rural areas in the Middle East from goats', ewes' and cows' milk. The product, which was evaluated by Tamime & Crawford (1984), was made from low fat milk, and the data for fat reported in Table 4 do not include olive oil in the analysis. A typical product should contain around 20% fat (Rosenthal *et al.* 1980); however, the authors should have named this product as labneh anbaris rather than labneh (see the difference in composition – Table 4).

Bulgarian buttermilk/sour milk or yogurt. A fermented milk popular only in Bulgaria, where *L. delbrueckii* subsp. *bulgaricus* starter is used alone (Marshall, 1984) in contrast to cultured buttermilks which employ mesophilic starters, but Bulgarian buttermilk has a sharp and clean flavour reminiscent of yogurt. Pasteurized whole milk is used and the incubation is carried out overnight at 40–42 °C to the desired level of acidity. In some instances *Str. salivarius* subsp. *thermophilus* is also included in the starter culture (Gyosheva, 1982, 1985; Gyosheva & Rusev, 1979). The volatile compounds (ppm) associated with the flavour of Bulgarian buttermilk are acetaldehyde (31·27–76·47), ethanol (21·20–75·00), acetone (24·13–58·28), 2-butanone (0·87–7·19), diacetyl (0·74–3·05) and ethylacetate (0–1·46) (Gyosheva, 1982).

In the USA, Bulgarian buttermilk is manufactured by mixing, at the time of breaking the curd, cultured buttermilk and milk fermented with *L. delbrueckii* subsp. *bulgaricus* in the ratio of 110 kg/2·4 kg respectively (Manus, 1979).

Dried yogurt products. Prepared in the Middle East by different methods. *Kishk*, for example, is a blend of yogurt or other related products (laban khad and/or laban zeer) and parboiled cracked wheat known in the region as burghol. The mixture is boiled, and the concentrated blend is shaped into 'rolls' of 10 cm in diam., then dried in the sun. The dried product is non-hygroscopic and has a shelf-life of up to 2–3 years; in Lebanon and Syria, the dried product is pulverized. Data regarding the compositional and microbiological quality are reviewed by Abou-Donia (1984). For consumption kishk is reconstituted in water, simmered gently over a fire and is normally consumed with bread. The consistency of this product is similar to porridge.

Madeer or *oggtt*, a similar product to kishk (without the added cereal), is made by the desert dwellers of Saudi Arabia from goats' and ewes' milk. The dried product is reconstituted in water and drunk like yogurt, or heated and eaten as a soup; it may also be consumed dry like a biscuit with dates. Research work to improve the quality of oggtt, which was manufactured from fresh milk or reconstituted milk powder, has been reported by Al-Mohizea *et al.* (1985) and Al-Ruqaie *et al.* (1987). The nutritional

quality of laboratory prepared madeer from cows' milk which was allowed to ferment at ambient temperature without the addition of a starter culture indicated significant amounts of mineral salts and other vitamins (Sawaya *et al.* 1984)

Dried yogurt is available on the market in the UK, but limited data are available on the process of manufacture. A typical chemical composition (%) of a semi-instant, low fat, dried yogurt is as follows: moisture 4, fat 4, protein 33, lactose 37, ash 7 and organic milk salts 11 (Anon. 1983*a*). The product is utilized in other food industries, including manufacture of baby food. The latter outlet, in the UK. was valued at £3·5 million in 1982 (Anon. 1983*b*).

Recent developments in yogurt manufacture

Several technological improvements have taken place recently in the industry.

Post fermentation heat treatment. This process aims to extend the shelf life of yogurt, as heating the product, for example at pasteurization temperatures, can destroy the starter bacteria and also contaminants such as yeasts and moulds. Inactivation of the yogurt microflora in the product may not be permitted in some countries, e.g. France. However, heating yogurt in its container to 58 °C for 5 min is sufficient to inhibit the activity of the yogurt bacteria. This process is known as heat shock (Driessen, 1984). Recently Waes (1987) evaluated this treatment for set yogurt and observed the total elimination of yeasts and that *L. delbrueckii* subsp. *bulgaricus* was more sensitive than *Str. salivarius* subsp. *thermophilus* to heat. He also noted that strain selection of lactobacilli is important if the viable count required after heat shock was to be above 10^5 cfu ml^{-1}. To obtain these results the acidity of the product should be ~ pH 4·6.

In some experimental work on heat shock yogurt (Driessen 1984), the product had been prepared in glass bottles rather than thermoformed plastics. Although the latter material is widely used, its industrial application for heat shock treatment is questionable. According to a survey in the UK, the reported level of styrene in yogurt ranged from < 1–200 μg/kg (Ministry of Agriculture, Fisheries and Food, 1983). The application of heat to yogurt in plastic containers could increase the level of styrene in the product, which may be undesirable to the consumer. Further research work is required in this area to determine the level of this substance.

Tampering with packaging. There has been increasing evidence of tampering with processed food sold in retail chain stores in Europe and North America. However, in the USA, Herner (1987) reported on consumer reactions towards 'tamper evident' packaging of cultured dairy products, and the demand that manufacturers guarantee the safety of their products. The most popular methods used for 'tamper proofing' are the shrink film overwrap, shrink bands and heat activated lids, and it is likely that similar approaches to protect the consumer will be adopted in the future on this side of the Atlantic.

New products. In 1986 the value of the yogurt market in the UK was estimated at £240 million (Anon. 1987*a*) which represents a market growth of 18%, and the projected figure for 1987 is £281 million. Ultimately, product development ensures the broadening of consumer appeal by the marketing of new yogurt products, such as yogurt with added cream, yogurt free from additives such as artificial colours, stabilizers and sugar, yogurt mousse and/or lightly whipped yogurt which is a blend of yogurt, cream, and fruit purée, and the use of fructose in fruit-flavoured preparations; aspartam as a sweetener in dietetic yogurt has also been developed.

Therapeutic products

These have been manufactured commercially in Europe, and are becoming popular with the health-conscious consumer. The organisms employed are thermophilic in nature, and the microflora used are the types isolated from the intestine of newly born babies, i.e. *Bifidobacterium* spp., or those that can become established in the gut i.e. *L. acidophilus*.

Bioghurt®, Biogarde® and Bifighurt®

These are cultures which can be incorporated into fermented foodstuffs for the production of cultured milks. The basic procedure of manufacture is shown in Fig. 2. The first stage is the propagation of the bulk starter. According to Rašić & Kurmann (1983), reconstituted skim milk powder at 11% TS is a convenient medium for starter propagation, and for strains which are difficult to culture 0·5% yeast extract may be included. The use of bulk starters rather than direct-to-vat inoculation (DVI) is recommended as a means both of reducing production time and of ensuring high numbers of viable cells in the end products. One of the problems with these products is that bifidobacteria are not competitive in the presence of other microorganisms, and even *L. acidophilus* is fastidious in its growth requirements. For this reason, the production of the bulk starter must take place under aseptic conditions (Tamime, 1981; Tamime & Robinson, 1985), with the constituent microorganisms being grown initially as monocultures (Schuler-Malyoth *et al.* 1968). The last authors proposed the following scheme for the production of a cultured milk containing bifidobacteria: monocultures of *Str. salivarius* subsp. *thermophilus*, *Bf. bifidum* and *L. acidophilus* were incubated for 7, 4 and 24 h respectively, all at 42 °C. A bulk starter containing the three cultures (ratio 1:1:1) was incubated for 4 h at 42 °C, then added to the milk and incubated for 3 h at 42 °C.

The relatively rapid fermentation time is a reflection of the high rate of inoculum required, i.e. 10–20%, in order to ensure that sufficient bifidobacteria are transferred; the end-product should contain 10^6–10^8 cfu ml^{-1} of *Bf. bifidum* (Schuler-Malyoth *et al.* 1968), and the increase during incubation is unlikely to exceed two log cycles. The final pH of the retail product must be above 4·6, because otherwise *L. acidophilus*, and more especially *Bf. bifidum*, will quickly decline in numbers. This requirement means that incubation must be terminated at pH 4·9–5·0 to allow for further acidification during cooling and packaging.

The precise figures for pH will, of course, depend on the strains involved, and certainly some isolates of *Bf. bifidum* show remarkable tolerance even of pH 4·0 (Rašić & Kurmann, 1983). However, it is probable that the commercial strains are far less acid-resistant, and assuming that a therapeutic minimum of 10^5 cfu ml^{-1} in a daily portion of 100 g should be present at the end of 14 d storage, then maintenance of a high pH becomes essential. Careful control of times and temperatures is therefore critical, but it is fortunate that the post-production characteristics of the Bioghurt® and Biogarde® cultures are different from those of a typical yogurt starter. Thus, while the production of lactic acid continues, albeit slowly, through the storage life of yogurt, in Bioghurt®, Biogarde® and Bifighurt® products the change in acidity is negligible at 2–4 °C (Klupsch, 1983). Consequently, there is no reason why these products should not retain their essential microflora for the duration of the expected shelf life.

Although the use of laboratory-grown cultures does offer certain advantages, in

other fields the use of commercially available starter cultures has become common practice. In the present context these latter cultures would be employed for the preparation of the bulk starter, and the sterile milk could be inoculated either with the individual species, or with a preblended mixture. The choice of one approach as against another is probably a matter of company policy, but the deep-frozen starter concentrates containing *Str. salivarius* subsp. *thermophilus* and *L. acidophilus* (with or without *Bf. bifidum*) provide excellent inocula for starter tanks.

The provision of starters as individual species does offer the potential user rather more flexibility in respect of the final ratio between the organisms, as well as perhaps opening the way to more exact control over the total numbers of bacteria in the retail product. Whether these reasons are important to the manufacturer will probably depend on the nature of the end product, but it has been reported that excessive numbers of bifidobacteria can impart a vinegar taint to fermented milks (Hansen, 1985). The use of single species may also render the cultures more amenable for DVI and this application is certainly intended for the so-called AB-cultures (see later).

The market growth of Biogarde® (Almhof brand) in Europe has been developing successfully over the past few years. In 1983 the annual production was 500 tonnes, and the figure soared to 7000 tonnes in 1986 (Anon. 1987*b*).

Marshall *et al.* (1982) have successfully grown *Bf. bifidum, adolescentis, infantis* and *longum* in ultrafiltered skim milk and in Cheddar cheese whey which had been concentrated ×2 and ×8 respectively. The viable counts of these bifidobacteria in fermented milks stored at 4 °C for 21 d ranged between $< 10^5$ and $> 10^8$ cfu ml^{-1}.

Acidophilus milk

This invariably includes viable strains of *L. acidophilus*, and the activity of this species has now been linked with the establishment of a normal gut flora in patients who have received antibiotic therapy (Nahaisi, 1986), the alleviation of habitual constipation (Graf, 1983), and reduction in levels of serum blood cholesterol in rats (Sinha *et al.* 1979).

The last two claims, as with suggestions of anti-tumour activity (Shahani, 1983), require much further investigation, but the *in vivo* competitive ability of *L. acidophilus* against potential pathogens is widely accepted (Sandine *et al.* 1972; Speck, 1976).

The reaction of the consumer to such claims has, even in the health-conscious United States, been rather less than enthusiastic, but acidophilus milk has found a limited niche in the market place. As a consequence, the dairy industry has sought ways of streamlining the traditional method of production, and a comparison of the original system and its modern variant is shown in Fig. 3.

Further reductions in the process time have been hindered by the fact that *L. acidophilus* grows only slowly in milk, and this milk has to be virtually sterile to avoid the proliferation of undesirable contaminants. Although a UHT process of 145 °C for 2–3 s could achieve this state (Chandan, 1982), few dairy plants have this facility available. A prolonged heating and cooling cycle is therefore the accepted solution. Also relating to the extended fermentation time, it is essential that the strain employed is capable of survival/implantation in the human intestine, and such biotypes are notoriously slow-growing. An overnight incubation of 12–16 h appears to offer the best solution, with an appropriate back-up system to monitor automatically the pH and initiate cooling at a preset level of acidity.

This latter point is critical, because to exhibit any of the expected therapeutic properties, the retail product must contain at least 500×10^6 cfu ml^{-1} of

L. acidophilus at the time of consumption (Speck, 1976). Survival at this level is directly dependent on the overall acidity of the milk, and if the acidity is allowed to rise above 0·6–0·7 % lactic acid, the viability of the cells will decline in a matter of days. Given adequate control over pH, then the required population level should be maintained for 14–21 d, but the extent of any success in this direction may well be influenced by the efficiency of refrigeration conditions of the retail chain. Partly to overcome the risk of reduction in viable cells, and partly to avoid the rather sour taste of traditional acidophilus milk, manufacturers have sought other ways of bringing products containing viable organisms to the consumer.

The most straightforward development has involved the preparation of a cell concentrate by centrifugation, followed by the addition of the cell mass to pasteurized market milk. This direct incorporation allows the manufacturer to adjust the cell count to an agreed figure, and because little acid development occurs under refrigerated storage the flavour is little different from that of normal milk. This sweet acidophilus milk has achieved considerable popularity in the USA, but in Europe the twin aims of stable cell numbers and improved flavour tend to have been pursued mainly by employing mixed inocula of *L. acidophilus* and other suitable species. The production of acidophilus yogurt is one obvious proposal that has been examined (Gilliland & Speck, 1977; Hull *et al.* 1984; Robinson, 1987), and provided that the cultures are carefully selected and the acidity is closely monitored, survival of *L. acidophilus* for at least 14 d seems to be assured. However, although this product meets the criterion of improved flavour, cell viability could still prove a problem in commercial practice.

An alternative has been to use *Bf. bifidum* as one of the inoculated species, a move of especial interest in light of the alleged therapeutic properties ascribed to this organism (Rašić & Kurmann, 1983).

Cultura-AB

A sour milk product that, in terms of consistency and flavour, resembles natural yogurt. The base is milk enriched to give a protein content of 3·8–3·9 %, and then heat treated in the same manner as yogurt milk. After cooling to 37 °C, the milk is inoculated with separate cultures of *L. acidophilus* and *Bf. bifidum*. If deep-frozen DVI cultures are used, then the inoculum rate is 250 g of *L. acidophilus* culture and 100 g of *Bf. bifidum* culture/1000 l milk. This slight imbalance ensures that the bifidobacteria do not become too dominant, for at the end of fermentation it is expected that the cell counts will be $2–4 \times 10^8$ cfu ml^{-1} and $1–2 \times 10^8$ cfu ml^{-1} for *L. acidophilus* and *Bf. bifidum* respectively (Anon. 1985).

The actual fermentation is a distinct contrast to that employing Bioghurt®, Biogarde® and Bifighurt® cultures, for Cultura is developed over a period of 16 h, and up to 18 h if lyophilized DVI cultures are used. Presumably the activity of *Str. salivarius* subsp. *thermophilus* is beneficial in accelerating acid development in the former products, but the pH of Cultura is allowed to fall to 4·1–4·2. At this level of acidity, the death rate of the constituent bacteria is extremely high, but even so it is reported that 'therapeutic minimum' counts are still present after 21 d at 7 °C (Anon. 1985).

Although Cultura can be produced in the form of a drink, it is basically a yogurt with a rather special microflora. The production plant requirements are therefore similar, but it is important that the incubation time is much extended. This means that, as with the long-set system for yogurt, the demands in terms of plant hygiene are comparably more strict.

Yakult

A therapeutic fermented milk product originating from Japan and with a high consumption rate there. It is also produced and distributed in the Far East and North and South America (Anon. 1986).

In 1984 130 tonnes of yakult and 352 tonnes of total lactic beverages were produced in Japan (S. Mitsuhashi, 1986, pers. comm.). The therapeutic properties of yakult are attributed to the high numbers of *L. casei*, which appear to behave in the human gut in a manner similar to *L. acidophilus* and *Bifidobacterium* (Anon. 1974). The precise details of the manufacture of yakult and related products are not readily available outside Japan. However, Table 5 illustrates the chemical composition of yakult and another similar product, compared with raw milk.

Yakult has a thin consistency and, to compensate for the junket-like characteristic, sucrose is added, presumably to enhance its organoleptic properties. The colour of yakult is slightly brown, which indicates that the milk/sugar base has been subjected to a high temperature before cooling and fermentation. Recently yakult flavoured with vegetable juices (e.g. tomato, celery, carrot, cabbage or parsley) has been marketed to provide the product with an entirely different image.

Yeast-lactic fermentations

Although many early fermentations were probably contaminated with yeasts, only in two instances does the growth of yeasts appear to have been consistently encouraged. Typical examples of such fermented milks are kefir and koumiss. The presence of yeast results in the production of ethanol and also of CO_2 which gives an effervescence characteristic to these products.

Kefir

Usually produced from whole cows' milk; after heat treatment (90–95 °C for 5 min) and homogenization, it is cooled to around 23 °C for the fermentation. Traditionally, the milk was then inoculated with a batch of so-called kefir grains, which consist of a complex population of yeasts and lactic acid bacteria embedded in a semi-solid matrix (Bottazzi & Bianchi, 1980). The precise identity of the constituent microorganisms has always been somewhat controversial, but the studies of Kandler & Kunath (1983) and Marshall *et al.* (1984) tend to suggest that only two organisms are intimately associated with the grains; these are *Candida kefir* and *L. kefir* (see also Engel *et al.* 1986).

Low fat kefir (1·5%) has been studied by Gawel & Gromadka (1978). The milk was heated to 85–90 °C for 10–15 min, cooled to 20 °C and 5% starter (kefir grains) was used for fermentation over one day with further ripening at 8–10 °C for another day. They concluded that the main biochemical changes in kefir occurred during the first day of fermentation, and that the organoleptic properties of the product were dependent on the activity of the kefir grains.

Multiple stage heat treatment of the milk (i.e. heating to 87 °C, cooling to 77 °C, re-heating to 87 °C, cooling to 77 °C and holding for 30 min) helps to increase firmness, elasticity and viscosity of the kefir curd, and reduce syneresis (Berzhinskas *et al.* 1978). UHT milk with 1 or 3% fat has been used for the manufacture of kefir, and the product has similar characteristics to kefir made from HTST milk (Merin & Rosenthal, 1986). Korovkina *et al.* (1978) recommended that fermentation of milk at 25 °C improves viscosity and flavour. The fortification of milk (1% fat) with 0·6% Na

Table 5. *Compositional quality (%) of yakult and a related product compared with raw milk*

Type of microorganism	Yakult *Lactobacillus casei*	Yakult Miru-Miru *Bifidobacterium bifidum* *Bf. breve* *L. acidophilus*	Average in raw milk —
Constituents			
Fat	1·1	3·1	3·6
Protein	1·2	3·1	3·2
Lactose	1·1	4·5	4·5
Other sugars	14·1	6·1	—
Ash	0·34	0·8	0·7

After S. Mitsuhashi, 1986 (pers. comm.).

caseinate is used for the production of a dietetic kefir called *osobyi* (Bogdanova *et al.* 1978).

In the absence of grains typical kefir can be manufactured with a wide range of lactic acid bacteria (Mann, 1985). One advantage of this approach is that it could allow the process to be handled as a two-stage fermentation, i.e. a lactic acid fermentation followed by an alcoholic one. Whether or not this division would offer benefits in relation to process control has yet to be established, but it would certainly allow operators to consider the use of DVI cultures rather than the sometimes temperamental kefir grains.

Koumiss

Produced traditionally from mares' milk, and although thousands of tonnes of true koumiss are manufactured annually in the USSR, a substantial volume is now based on whole cows' milk, or a mixture or skim milk and cheese whey, with or without added cream (Mann, 1985).

The basic process involves a heat treatment of the milk to 90–92 °C for around 5 min followed by homogenization if appropriate, and cooling to 26–28 °C. The inoculum, which may range from 10–30%, probably consists of a mixture of *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Kluyveromyces lactis* (Robinson & Tamime, 1981), and the length of fermentation is adjusted to provide an end-product with acidity from 0·6 to 1·0% lactic acid and ethanol content of 0·7 to 2·5%. At the selected end-point, the coagulum is agitated vigorously to a smooth consistency, and then packaged.

Recently, Puhan & Gallmann (1980) developed a method for the production of koumiss from UF modified cows' milk. The process involves mixing five parts of whole cows' milk with eight parts of UF concentrated rennet whey (2:1), this modified milk being similar in composition to mares' milk. The processing stages are similar to yogurt until the inoculation stage. At this point, β-galactosidase is added together with a yogurt starter culture (1·5%), and at pH 4·4, the coagulum is cooled to 20 °C. The product is then inoculated with a koumiss yeast culture (4%), incubated for 6 h in a stir bottle (with crown cork), and cooled to 5 °C for storage. This method of manufacture claims to minimize the separation of the two phases of milk; however, owing to the build-up of CO_2 pressure in the bottle, this type of koumiss tends to foam on opening.

The addition of *Acetobacter aceti* to a koumiss starter culture containing *Lact.*

lactis subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus* and the yeast *Torula lactis* has been reported as a new starter culture for koumiss, and is alleged to mimic the specific aroma of the product made from mares' milk (Shigaeva & Ospanova, 1982).

At present, there are few indications that koumiss is proving acceptable outside the USSR, but the emergence of new brands, including one produced from camels' milk (Belokobylenko, 1984) suggests that the product could acquire a wider appeal.

Acidophilus–yeast milk

A fore-runner of a yeast–lactic fermented milk product which could be developed for the European and North American markets. Subramanian & Shankar (1985) achieved high numbers of viable cells of *L. acidophilus* ($6{\cdot}2–9{\cdot}8 \times 10^8$ cfu ml^{-1}) in the presence of lactose-fermenting *Saccharomyces fragilis* or *Candida pseudotropicalis*.

The milk is heated to 90 °C for 20 min, and coagulation achieved in < 20 h at 33 or 37 °C. The consistency of the coagulum is improved by fortification of the milk with 1·5% skim milk powder and 0·5% agar. The latter compound prevents the break-up of the curd caused by the production of CO_2.

The packaging of yeast–lactic fermented products in hermetically sealed laminated paper board cartons or plastic containers may pose the problem of consumer rejection of swollen packages due to a build-up of pressure as a result of CO_2 formation inside the container; recently a breathing membrane which allows CO_2 to escape has been developed in Switzerland (Flückiger, 1986).

Mould–lactic fermentation

Viili

A cultured milk product from Finland. The two different types of viili are the natural or plain-flavoured viili (3·9% fat), also *kevytviili* (2·5% fat) and the sweetened and fruit-flavoured *marjaviili* (Anon. 1982).

The standardized milk (3·9 or 2·5% fat) is heated to 85–90 °C without homogenization, cooled to 20 °C, inoculated with the starter culture, packed into cups, incubated at 18–20 °C until the acidity is 0·9% lactic acid and finally cooled. During the incubation period, the fat rises to the surface where the mould *Geotrichum candidum* grows to form a velvet-like layer on the product (see also Meriläinen, 1984).

The introduction in the early 1980s of marjaviili, which has a layer of fruit in the bottom of the cup, has increased consumption of this type of fermented milk in Finland by 10%, and the annual total consumption of viili is 6 l *per capita* (Meriläinen, 1985).

Miscellaneous fermentations

Numerous rural processing methods have been used by man for the manufacture of different fermented foods in the developing countries (for a review of these indigenous foods see Steinkraus, 1983). The products, which could be of potential interest in expanding the fermented milks market in the developing countries, are mainly formulated using mammalian milk plus non-animal protein products. This approach is attractive in countries where there is shortage of milk. Some published data on the use of soyabean flour, peanut flour or leaf protein in the yogurt industry have been reported by Robinson & Tamime (1986*a*); however, further trials must be

carried out in order to improve the acceptability of such proteins. Recently, *gata* (coconut milk) has been used in the Philippines for the production of yogurt (Sanchez & Rasco, 1984). The recommended formulation is coconut milk blended with reconstituted skim milk powder in a ratio of 50:50, heating to 85 °C for 20 min, cooling to 42 °C, inoculation with 1·5% *Str. salivarius* subsp. *thermophilus* and incubation for 30 min before the addition of 1·5% of *L. delbrueckii* subsp. *bulgaricus*; fermentation takes place in the container to the desired pH (4·5–4·7), with final cooling and storage. The same authors concluded that the consistency of the product is improved by addition of extra skim milk powder to the basic mix and 12% sugar.

METHODS OF STARTER CULTURE PRODUCTION

The distinctive features of the various products discussed above are derived in large measure from the metabolic activity of the specific starter cultures. This association means that the correct species, and usually strain of that species, has to be added to the process mix in an active, viable state, and in sufficient numbers to complete the desired fermentation within a set process period. Where a predetermined mixture of species is required, then the numerical balance between the organisms must also be achieved. Some of the methods employed to satisfy these demands are tabulated here (Table 6).

The final choice of any given system of inoculation will depend on a number of factors, such as the location of the plant in respect of culture suppliers, the sophistication of any on-site laboratory and, perhaps, the nature of the final product, but whatever the ultimate selection, the demands of a production facility can usually be met. Nevertheless, product manufacturers are relying increasingly on specialists for the routine supply of cultures. The trend away from in-house production is largely because commercially produced starters give more consistent activity than liquid cultures subject to daily transfers; they also make less demand on specialist laboratory staff, allow easy rotation of cultures to avoid the build-up of phage, introduce the possibility of change to DVI for the process milk and lower the risk of infection by adventitious organisms.

For products with complex and/or ill-defined starter cultures, e.g. koumiss, this transition may not be possible at present. However, the proposal that kefir can be made with a precisely defined mixture of species, each added separately to the process milk, does suggest that even traditional fermentations could be adapted to incorporate the latest developments in starter technology.

FUTURE DEVELOPMENTS

The manufacture of any fermented food is something of a balance between the traditional art and modern science, and the production of the various milks described above is no exception. The relative importance of tradition and technology varies, so that while yogurt making is now a highly automated process, other procedures are little more than scaled-up versions of their cottage origins. The reason for this disparity lies mainly with differences in consumer demand, for there is little incentive for investment in a product with comparatively low sales. This same basic pattern will, no doubt, be followed in the future and hence the extent to which consumers will adopt new flavours and/or types of product will largely determine the rate and direction of future developments. It could be argued, however, that economic forces

Table 6. *Summary of the alternative forms of starter culture that are employed for the manufacture of fermented milks*

Physical state of culture	Facilities required to produce bulk starter	Inoculation level into process mix	Example of application
Liquid	Laboratory to propagate stock and mother cultures, intermediate and bulk starter vessels (for details see Tamime, 1981)	1–3% (v/v) 3–5% 10–30%	Yogurt Kefir Koumiss
Frozen			
Short-term (−40 °C)	Intermediate and bulk starter vessels	As above	Yogurt
Long term (−196 °C)	Bulk starter vessels or Inoculation directly into the process mix	1 can/1000 l	Yogurt Acidophilus milk
Lyophilized	Bulk starter vessels or Inoculation directly into the process mix	1 sachet/1000 l	Yogurt Acidophilus products with or without *Bifidobacterium*

and centralization of the production of fermented milks, and in particular yogurt, may help re-introduce, for industrial purposes, the continuous yogurt process which was developed a few years ago by the collaboration of the Netherlands Institute for Research in Dairying and Stork–Amsterdam in Holland (see Tamime & Robinson, 1985).

The growing interest in products with a health-promoting image has encouraged starter manufacturers to offer a range of cultures, including *L. acidophilus* and *Bf. bifidum*, and to ensure that these cultures have patterns of growth and survival that satisfy both the manufacturer and the health-conscious consumer. The fact that a conventional yogurt unit can be employed for production means that, at least initially, manufacturers of plant and equipment will be under no pressure to design any specialized items, but the development of multi-stage fermentations, as proposed for kefir, could necessitate some degree of innovation.

However, if plant manufacturers are able to indulge in a measure of conservatism, the suppliers of cultures certainly cannot. Already the trend towards DVI cultures is posing demands for starters of unchanging performance, and future requirements may prove even more stringent. In particular the potential of genetic engineering has raised the prospect of 'ideal' cultures, and certainly the derivation of mesophilic strains that are totally phage resistant is a clear possibility (Huggins, 1984). Strains of lactic acid bacteria with enhanced levels of metabolism in respect of a specific pathway are also being sought, and technically at least the outlook is encouraging. This optimism must be balanced, however, by the realization that such cultures would be employed in food products and, as pointed out by Barach (1985), what happens to the harmless status of a starter organism once it has been subjected to genetic engineering? To date, the regulatory authorities have not become formally involved with the clearance of a specific culture, and the resolution of the first test case will be watched with interest.

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Freeze-dried starter concentrates part 1

Their characteristics and potential application to the production of cheese and yoghurt

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The production of an active contaminant-free bulk starter is the corner-stone of any process leading to the successful manufacture of a fermented dairy product, and hence it is not surprising that this area of creamery operation has long been a source of concern to the production manager. Originally this concern would have prompted an involvement in all stages of starter preparation within the creamery, but in recent years, this pressure has slowly been reduced, and principally through the endeavours of the independent manufacturers of starter cultures.

Initially, these efforts were limited to the supply of stock cultures, or perhaps inocula for the direct preparation of a mother culture, and although the introduction of these new cultures was welcome, the creamery was still left with the onerous task of building up the volume to provide the bulk starter. It was perhaps inevitable, therefore, that the dairy industry and its suppliers began to speculate about the possibility of cultures that could be inoculated directly into the process milk itself, and the results of this speculation are outlined in Table 1.

Each one of these systems enjoys the support of its adherents, but it is noticeable that an increasing number of dairies are turning to the specialist suppliers of starter cultures for their needs. Thus, the ever rising cost of maintaining on-site culture facilities, together wth the constant risk of starter failure, has rendered the production of liquid or similar cultures increasingly unattractive, but it is the introduction of 'off-the-shelf' concentrated starters that has really tipped the balance in favour of the starter manufacturers.

Freeze-dried starter cultures

The process of freeze-drying (lyophilization) is widely employed for the preservation of biological materials, particularly in situations where the inherent characteristics of the material must be maintained unaltered, and it is this technique that has now been applied with great success to the preservation of bacterial cultures (see Figure 1.). Some forms are intended merely to replace the traditional stock-cultures held by a laboratory, but the freeze-dried concentrated starters represent a specific response to the need for cultures that are suitable for direct inoculation into either the bulk starter tank, or alternatively, into the process milk itself. At first sight, these two methods of using the cultures appear similar, but in practice, the situations are quite different, and in a number of important respects:

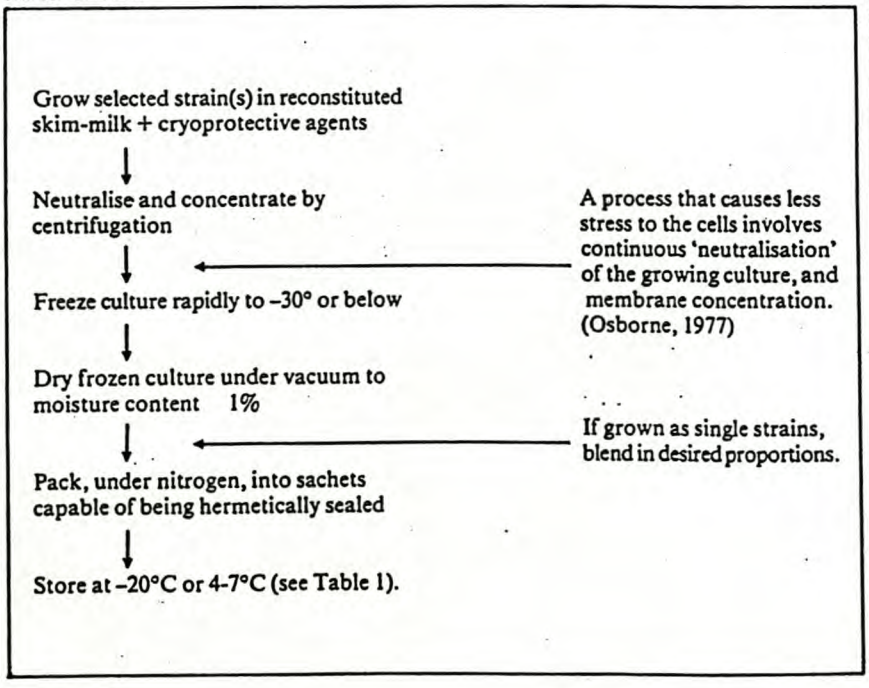
i) the milk in a bulk starter tank will be virtually sterile, and hence the starter organisms should be subject neither to competition from other bacteria, nor to infection by phage; the milk for processing into cheese will only be pasteurised, and may contain both bacteria and phage particles;

ii) the comparatively small volumes of medium required for starter production allow for adjustment of the chemical composition of the milk to encourage maximum culture activity, whereas the cultures employed for direct-to-vat inoculation have to show optimum growth and metabolism in milks of widely differing composition;

iii) the schedule for starter production is normally more flexible than that for processing, so that while a freeze-dried starter for direct-to-vat usage will be expected to perform as well as its liquid counterpart, some degree of tolerance will be accorded to cultures giving rise to a bulk starter.

It would be quite unrealistic, therefore, to assume that a thousand gallon vat of process milk simply requires a rather larger inoculum than a hundred gallon starter tank, and indeed the success of the direct-to-vat freeze-dried concentrates is an indication that they represent a new concept in starter formulation. For this reason, it is appropriate to consider the available cultures in relation to their intended mode of use, for it is pressure from this source that has, perhaps above anything else, dictated the characteristics of the starters concerned.

Figure 1. An outline of an hypothetical procedure for the production of a freeze-dried starter culture.



The Production of Bulk Starters

The freeze-dried concentrates intended for direct inoculation of bulk starter vessels are distributed by Chr. Hansen's Laboratorium A/S, and are sent through the mail in light-weight, aluminium pouches. Each individual sachet contains sufficient bacteria to inoculate a specific volume of milk, and in the case of Cheddar or other cheeses, the volumes are 45 gallons or 220 gallons. The equivalent cultures for yoghurt are designated as meeting bulk starter requirements for 110-220 gallons, but larger volumes can, of course, be handled simply by employing more than one sachet.

The procedure for producing a bulk starter is quite straightforward, and consists of the following steps:-

Heat-treatment of medium (as existing operation)

↓

Cool to incubation temperature

↓

Surface 'sterilize' sachet with alcohol

↓

Cut open, and pour contents into starter vessel

↓

Agitate to ensure even distribution of culture

↓

Incubate to desired acidity

and it is clear that if this approach is contrasted with the traditional system involving serial inoculations of liquid cultures, then it represents a most efficient and inexpensive method of starter production. The cultures offer the additional attraction that they can be stored for 5-6 months at -20°C without loss of activity, and hence the costly storage facilities required for the deep-frozen concentrates are quite unnecessary.

For the manufacture of Cheddar or similar cheeses, there are now some fifteen cultures available containing mixed populations of *Streptococcus cremoris* (<95%) and *Streptococcus lactis* (>5%), and sufficient phage-unrelated strains of *Str. cremoris* to provide a 5-day rotation. The fact that these same strains have been employed for many years in the industry implies that the finished cheese should be of a quality identical with that normally associated with the plant in question, and this confidence has certainly been justified in respect of the equivalent yoghurt cultures. Thus, yoghurt manufacturers tend to require cultures capable of producing either a firm, clean-cut coagulum with a pronounced, aromatic flavour (Set Yoghurt), or a coagulum that is resistant to agitation and the incorporation of fruit (Stirred Fruit Yoghurt), and this basic choice can be accommodated from the cultures on offer.

The only significant difference, therefore, between a liquid culture and this type of freeze-dried concentrate is that the bacteria in the latter are in a state of 'suspended animation', while those in the former are, in terms of their metabolism, fully active. This contrast means that, although the viability of the freeze-dried cultures is excellent and the total number of bacteria added to the bulk starter milk may be the same in both cases, the time required to produce a bulk starter will be increased. This effect is illustrated in Figure 2, and assuming that the desired acidity for a bulk starter for yoghurt is around 0.85% lactic acid, ie. both *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in their logarithmic phase of growth, then a

Table I. The alternative forms of starter culture that are available, and some observations on the method of utilisation

Type of Culture	Comments
Liquid Cultures	Expensive in terms of laboratory facilities; Requirement for bulk starter and intermediate culture vessels; Demanding on creamery personnel; High risk of infection during transfers, eg. bacteria, yeasts or phage; Culture characteristics liable to change with frequent sub-culturing.
Frozen cultures a) Short-term at -20 to -40°C eg. Cheese Starters (Dairy Cultures Ltd.)	Requirement for central laboratory within company, but also available from independent suppliers; Requirement for rapid and reliable transport service; Requirement for bulk starter (and perhaps intermediate) culture vessels; Low risk of infection if bulk starter facilities are well maintained; Less demanding on creamery personnel; Improved control of cultures should eliminate undesirable changes.
b) Long-term at -196°C eg. Marstar Cultures (Miles Laboratories Ltd) Redi-set Cultures (Chr. Hansen's Laboratorium A/S).	Expensive culture storage facilities required; Requirement for bulk starter vessels;* Easy to use; Low risk of infection; An extensive spectrum of phage unrelated strains is available for cheese production, but plans for culture rotation can become complex. *Some cultures, eg. Hansen's DVS Cultures, can be employed for direct-to-vat inoculation.
Dried Cultures a) Freeze-dried (standard) eg. Dri-Vac Cultures (Chr. Hansen's Laboratorium A/S	Eliminates need for maintenance of stock cultures; Standard culture characteristics; Otherwise handled as liquid cultures (see above).
b) Freeze-dried (concentrates) i) Bulk starter production eg. Redi-set Cultures (Chr. Hansen's Laboratorium A/S)	Requirement for domestic deep-freeze (-20°C) for storage; Requirement for bulk starter vessels; Low risk of infection, and rotation of phage unrelated strains employed in cheese plants; Easy to use; Can be employed as direct-to-vat cultures, and have proved especially useful for small scale production of yoghurt, but procedure would be too expensive for large creameries.
ii) Direct-to-Vat System eg. ICF Cultures (Eurozyme)	Requirement for domestic refrigerator (7°C) for storage; No requirement for bulk starter vessels; Low risk of infection; Rotation of cultures for cheesemaking on weekly basis; Estimated cost per tonne of cheese is reported as "comparable to to the production of a liquid bulk starter".

traditional bulk starter should reach this stage in 2½-3 hours at 42°C, but starter milk inoculated with a freeze-dried culture would not reach this level of acidity until 4-4½ hours. This delay is simply confirmation that the freeze-dried cells need a longer time to attain optimum physiological performance, and it must be emphasised that the activities of the final bulk starters will be indistinguishable.

The extended lag phase apparent in Figure 2 need not be important in practice, for little more than a slight modification to the schedule of starter production will totally nullify any inconvenience that could arise from the initial sloth of the culture. Indeed, once the new 'time-table' has become operational, the use of freeze-dried concentrates can greatly simplify the tedium of bulk starter production, and it is no surprise that their popularity is increasing rapidly.

Nevertheless. the creamery is still left with the demand for expensive bulk starter tanks, the cost of the starter medium, the energy costs of heating the medium, and the need for personnel to monitor the entire operation, and hence it is only natural that cheesemakers should have retained their dream of the 'ultimate starter', namely an inexpensive, reliable, 'off-the-shelf' culture for direct addition to the process milk. Whether this dream will ever become reality remains to be seen, but there can be little doubt that the latest addition to the 'field' offers a real prospect of success.

Direct-to-Vat Inoculation

The demands made upon a starter culture employed for direct-to-vat inoculation are rigorous, in that:

i) it has to be totally reliable;
ii) it has to perform efficiently irrespective of normal seasonal or other variations in milk quality;
iii) the quality/Grade of the end-product must be comparable to that obtained with the starter system already in use;
iv) the cost to the cheesemaker must be no greater, *per* unit weight of cheese, than the production of a conventional liquid bulk starter,

and it was against this background that the special freeze-dried concentrated starters for direct inoculation were developed. At present, cultures of this type are marketed by one company, Eurozyme, and it is worth considering how far the restraints imposed by the manufacture of a hard-pressed cheese, eg. Cheddar, can be met by these ICF (Inoculum pour Cuve de Fabrication) cultures.

i) Reliability of the Cultures

The reliability of a culture depends, in the first instance, on the selection of strains of bacteria that are resistant to freeze-drying and on choosing the optimum conditions for the actual freezing and drying operations, for once this groundwork has been completed, it should be possible to produce cultures on a regular basis with little variation in their activity or viability. This latter point can, of course, be confirmed by the imposition of an adequate system of quality control, and a typical specification for an ICF culture would be:

Starter organisms	2×10^{11}-10^{12} colony forming units (cfu)/g.
Coliforms	<5 cfu in 1g.
Moulds	<10 cfu in 1g.
Yeasts	<10 cfu in 1g.
Activity	1 Unit/g*

*A Unit is the quantity of freeze-dried culture required to produce 150 m.mols. of lactic acid in skim-milk (10% total solids) in 4 hours at 30°C. (Anon, 1981).

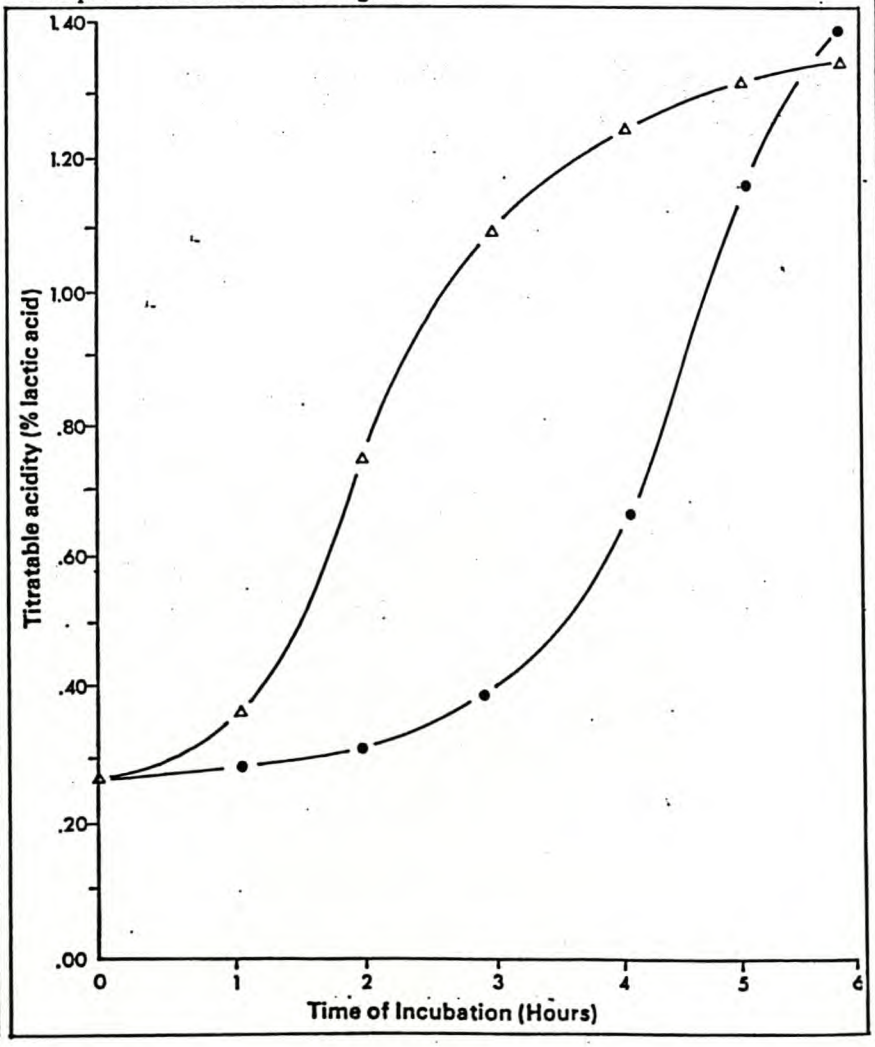
The application of these standards ensures that the cheesemaker is always working with a culture of known activity, and hence the problems caused by over-ripe bulk starters (Pearce & Brice, 1973), or starters weakened by phage attack, are eliminated.

The working cultures are available in standard sachets containing 10, 20, 50 and 100 Units, and the rate of usage depends on the type of cheese being made and, to a lesser extent, on the process/plant in question. However, in general a hard cheese, eg. Cheddar, would be inoculated at a rate of 40-45 units/250 gallons of milk (Chapman, 1978), while soft cheeses such as Carré de L'Est or Camembert require rather lower levels, namely 20 units/250 gallons and 0.5 units/250 gallons respectively (Mietton *et al*, 1978; Vassal *et al*, 1978).

ii) Performance during Cheesemaking

Three factors are mainly responsible for the phenomenon of poor acid development during cheesemaking, namely, (a) an over-ripe or poorly balanced starter, (b) infection by phage, and (c) low quality milk containing, perhaps, antibiotic or other residues, and any direct-to-vat starter must be able to cope satisfactorily with these pressures. The first problem has, of course, been dealt with by the very nature of the starter, and the essential question is, therefore, whether an ICF

Figure 2. This graph shows the typical development of acidity in reconstituted skim milk (16% T.S.) employing (△) a liquid culture and (●) a freeze-dried culture of Streptococcus thermophilus *and* Lactobacillus bulgaricus.



culture can be formulated to accommodate the vagaries of normal process milk.

In theory at least, the answer must be affirmative, because the freeze-dried concentrates marketed by Eurozyme are based on the multiple strain concept pioneered by Limsowtin *et al* (1977). In this work, six phage unrelated strains of *Str. cremoris* were assembled into one culture, along with a low level of *Str. lactis*, and the combination was employed daily for up to eight months in three different commercial creameries manufacturing Cheddar Cheese. It is the translation of this highly successful concept into a practical system for the cheesemaker that has now been achieved by Eurozyme, with the additional safeguard that two multiple strain combinations are available to give a weekly rotation of phage unrelated cultures, and some recent trials at the National Institute for Research in Dairying (NIRD), Reading, have confirmed this initial optimism.

In the work at the NIRD, two multiple strain, freeze-dried starters manufactured

Plate 1. After calculating the number of Units required for the volume of milk in the vat, the inoculation procedure is straightforward.

by Eurozyme (Code No. S11 and S12) were each used on eight occasions to make Cheddar cheese. Single herd bulk milk was used either fresh (mixed evening and morning milkings) or after storage at 4°C for 24 hours or 48 hours, and after pasteurisation (72.5°C/17 sec.), the milk was cooled to rennet temperature (32°C) and poured into the pilot-scale vats (45 gallons). One sachet of culture (10 Units) was then added to each vat (see Plate 1), and the cheeses made along the lines described by Chapman (1978).

The results are shown in Table II and it is clear that, apart from the slight elevation of the temperature at renneting, the process is essentially similar to that normally employed for the manufacture of Cheddar cheese. The maximum rennet to mill time of 5½ hours is, of course, longer than would be expected in the presence of a traditional starter, but even so, the delay is within the limits of 'normal practice'.

It is also notable that these new ICF cultures are a considerable improvement on their forebears, and that, in particular:

i) the prolonged lag phase usually observed during the resuscitation of freeze-dried cultures has been curtailed, and hence the rate of acid production in the vat is approaching that recorded with a liquid inoculum (Chapman, 1978);

ii) possible synergism between the constituent strains appears to enable the culture to cope with variations in milk supply;

iii) the preliminary trials have not indicated any susceptibility to phage present in the process milk;

iv) although use of the ICF cultures necessitates minor adjustments to the cheesemaking process, eg. a higher temperature at renneting, the quality of the finished cheese is comparable to that produced with a conventional bulk starter.

Table 2. Production records for the manufacture of Cheddar cheese employing ICF cultures — No. S11 and S12 — Each culture was used on eight occasions and where a spread of data is indicated, the figures represent the extremes of variation.

	Culture S11 Time/Temperature	Acidity (% lactic acid)	Culture S12 Time/Temperature	Acidity (% lactic acid)
Ripening time	25-30min.	—	25-30min.	—
Rennet to cut	40min.	0.10	40min.	0.10
Rennet to scald (max.)	2h.05min.-2h.10min.	0.12	2h.05min.-2h.15min.	0.12
Rennet to whey drainage (finish)	3h.30min.-4h.00min.	0.20-0.22	3h.20min.-3h.50min.	0.21-0.23
Rennet to mill	5h.05min.-5h.30min.	0.55-0.60	5h.05min.-5h.30min.	0.57-0.62
Inoculation to Press	5h.55min.-6h.30min.	0.66-0.72	5h.25min.-6h.20min.	0.67-0.70
Renneting temperature	32°C		32°C	
Scald temperature	39°C		39°C	

Based on: Chapman (1981) — unpublished data.

Conclusion

There is no doubt that freeze-dried cultures provide, in terms of convenience and reliability, the ideal inoculum for a bulk starter (Anon, 1980), but whether similar cultures can be employed for direct addition to the process milk is still a matter of some controversy.

The recent trials carried out in the UK, as well as those reported by Mietton *et al* (loc. cit) and Vassel *et al* (loc. cit), indicate that a range of cheeses, including Cheddar, Carré de l'Est and Camembert, can be successfully manufactured employing freeze-dried concentrated starters, and furthermore, that the time-consuming, and often extremely demanding, procedure of starter production can now be reduced to just one simple operation-emptying the contents of a sachet into the process milk. If these conclusions are a fair assessment of the situation, then the cheese industry is going to be forced to consider the implications very seriously, because it appears, on present evidence, that the cost of the new system will, *per* unit weight of cheese, be highly competitive.

Nevertheless, the final accolade, if indeed there is to be one, must come from the industry itself, for unless the cultures can prove themselves under normal commercial conditions, and over a long period of time, then creamery managers will not be tempted from their normal practice. It was with this need in mind that, earlier this year, Eurozyme invited a number of creameries to introduce direct-to-vat freeze-dried concentrates into their normal cheesemaking procedures, and it is the performance of these starters in a typical industrial climate that will be discussed in a second article to be published later this year.

Acknowledgement

The author would like to thank Miss Helen Chapman for her help in the preparation of this paper, and for making available the results of the experimental trials conducted at the NIRD.

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Freeze-dried starter concentrates part 2

Their use as direct-to-vat starters in the manufacture of Cheddar cheese

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THE essential characteristics of a starter culture employed for the direct-to-vat inoculation of process milk are that:

i) It has to be totally reliable;
ii) It has to perform efficiently irrespective of variations in the quality of the incoming milk;
iii) The quality of finished cheese must be acceptable to the cheesemaker, as must the cost of the culture *per* unit weight of cheese,

and the extent to which the ICF (Inoculum pour Cuve de Fabrication) or 'Ezal' cultures marketed by Eurozyme might meet these requirements has been discussed in the October issue of this journal (Robinson, 1981). However, the data presented on that occasion was based on trials performed in an experimental dairy, and while the results were most encouraging, the conditions were clearly less demanding than would be encountered in a commercial creamery.

For this reason it was decided to offer the cultures to two farmhouse cheesemakers, for if the starters could satisfy the exacting standards of this type of operation, then it would provide clear evidence that this method of direct-to-vat inoculation represents a truly practical proposition.

Performance in the Creamery

The principal site for these trials was a small factory in Somerset. The normal intake of this plant is 4,000 gallons of milk/day, and this supply is used to make Cheddar cheese in 2 × 2,000 gallon vats (Wincanton Engineering Ltd.). The process itself is entirely traditional, except that the final stage(s) is completed in a Wincanton Block Former. The finished blocks are then stored at 10°C for maturation prior to grading within the Farmhouse Scheme.

The practice of employing two vats in parallel made it an ideal venue for the trial, in that while one vat was being inoculated with the usual liquid bulk starter, an ICF culture could be added to the other. It was possible, therefore, to monitor the performance of the ICF cultures under normal routine conditions, and these observations occupied an eight-week period during May and June.

During each week, an ICF culture was employed on at least two occasions, and in order to monitor their comparability, two multiple-strain starters (Code No. S11 and S12) were used alternately. The first two weeks provided, in addition, an opportunity to standardise certain process parameters, and the cheesemaking times/acidities recorded over this period are shown in Table 1. A typical control schedule, ie. one involving inoculation of the milk with a liquid bulk starter, is included for comparison, and this association serves to highlight certain aspects of the process, in that:

i) An inoculation rate above that recommended for Cheddar cheese, ie.

Table I Some making times and acidities recorded during the manufacture of Cheddar Cheese employing the freeze-dried ICF starters from Eurozyme; the control times are typical of those recorded in the presence of a liquid bulk starter. The standard procedure provides an indication of the times that could be anticipated on a typical process day.

Process	Control	Trial with ICF Cultures				Proposed Standard Process (ICF Cultures)
Vat No.		(1)	(2)	(3)	(4)	
Culture	Liquid bulk starter	S11	S12	S11	S12	S11 or S12
Rate of Inoculation *(units/100 litres)*	2%	4.9	5.5	5.5	5.5	4.5-5.0
Ripening temperature	30°C	32°C	32°C	33°C	33°C	33-34°C
Ripening time	20-30 min	25 min	45 min	50 min	50 min	30 min
Rennet to cut	30 min	45 min	50 min	40 min	35 min	40 min
Rennet to 'heat-on' *(approx)*	1h 00 min	50 min	1h 00 min	45 min	50 min	1h 10 min
Rennet to scald *(max)*	1h 40 min	1h 30 min	1h 40 min	1h 25 min	1h 30 min	1h 50 min
Rennet to whey drainage *(start)*	2h 15 min	2h 30 min	2h 40 min	2h 25 min	2h 30 min	2h 50 min
Rennet to whey drainage *(finish)*	3h 15 min	3h 30 min	3h 35 min	3h 15 min	3h 25 min	3h 30 min
Rennet to mill	4h 30 min	4h 50 min	5h 20 min	4h 45 min	4h 40 min	5h 00 min
Inoculation to press	5h 30 min	5h 45 min	6h 35 min	6h 05 min	6h 00 min	6h 00 min
Acidities *(% lactic acid)*						Expected Acidities *(% lactic acid)*
at cutting	0.15	0.13	0.13	0.13	0.13	0.12-0.13
at max. scald	—	0.14	0.14	0.13	0.15	0.13-0.14
at whey drainage *(finish)*	0.36	0.23	0.21	0.26	0.31	0.23-0.26
at milling	0.65	0.55	0.64	0.61	0.64	0.56-0.60

5 units/100 l of milk, did not reduce the making time;

ii) Elevating the ripening temperature to 33/34°C markedly increased the rate of resuscitation of the bacteria in the freeze-dried starters, but extending the ripening time beyond 30 minutes had no demonstratable effect;

iii) This 'encouragement' of the starter towards maximum physiological activity was further enhanced by stirring the vats, for at least 25 minutes, after cutting, at 33°C prior to heating to the required scalding temperature;

iv) The initial rate of acid production was slower with the ICF cultures than with the active bulk starter, and this difference was reflected in longer "rennet to cut" times; commercial rennets, including the microbial rennet — Marzyme II — employed in this trial, are sensitive to small changes in acidity. However, the acidities of the curds at milling were entirely comparable;

v) Although the average rennet to mill time associated with the ICF cultures was somewhat longer than the typical 'control', the overall process time (inoculation to press) of 6 hours would be widely acceptable in commercial practice.

On the basis of this preliminary work it was possible to draw-up a tentative 'making schedule' (see Table 1), and these times provided the background against which the remaining six weeks of the trial were planned. The necessary flexibility of the cheesemaking process, in the light of variations in milk quality and/or other conditions, was accommodated in the normal way, and it became rapidly evident that the ICF cultures from Eurozyme could form an effective alternative to the traditional liquid bulk starter. Final confirmation of this assessment came when the cheeses were presented to the Official Grading Scheme at twelve weeks. Thus there was, at this time, no discernible difference at all between cheeses made with ICF cultures and those made with the normal starter — a clear indication that multiple-strain starters can produce excellent cheese, irrespective of whether the inoculum is liquid (Limsowtin *et al*, 1977) or in a freeze-dried form. Indeed so promising was the behaviour of the ICF cultures, that a neighbouring producer of Farmhouse Cheddar cheese undertook to establish a similar programme of comparative assessments.

The performance of the ICF starters during this second trial was, in all essential respects, similar to that recorded during the final six weeks of the earlier run, and the data shown in Table II confirm the extent to which the cultures can perform with predictable efficiency under an entirely different regime of cheesemaking. It is also relevant that the two creameries involved with these field trials are served by entirely different collection areas in respect of their bulk milk supplies, and hence that the balance of strains within the ICF cultures does enable them to cope with milks of different origin and/or chemical composition.

Table II The making times recorded over a two week period after the 'standard process' for employing ICF cultures had been introduced into a second creamery. Although the total process times were, as anticipated, slightly longer than normal, the consistent behaviour of the starters was impressive.

Process	Trial with ICF Cultures			
Vat No.	(1)	(2)	(3)	(4)
Culture	S11	S12	S11	S12
Rate of Inoculation *(units/100 litres)*	5.3	5.3	5.3	5.3
Ripening temperature	33°C	33°C	33°C	33°C
Ripening time	30 min	30 min	30 min	30 min
Rennet to cut	45 min	50 min	45 min	35 min
Rennet to 'heat-on' *(approx)*	1h 15 min	1h 20 min	1h 15 min	1h 05 min
Rennet to scald *(max)*	1h 55 min	2h 00 min	1h 55 min	1h 45 min
Rennet to whey drainage *(start)*	2h 55 min	3h 00 min	2h 55 min	2h 45 min
Rennet to whey drainage *(finish)*	4h 05 min	3h 45 min	3h 45 min	3h 55 min
Rennet to mill	5h 00 min	4h 55 min	5h 00 min	5h 00 min
Inoculation to press	6h 00 min	5h 55 min	6h 00 min	6h 00 min

Prospects for the Future

It was suggested earlier that the potential of direct-to-vat freeze-dried starters could only be assessed in the light of their performance in a commercial environment, and it must be conceded that the ICF cultures from Eurozyme have met this requirement with comparative ease. Thus once the process conditions had been stabilised for a given plant, the making times were only marginally longer than those recorded employing a traditional liquid inoculum. Equally important is the fact that the quality of the finished cheeses was consistently high, and hence the prediction of Limsowtin *et al* (1977) that the 'multiple-strain' concept would, on refinement, provide the basis for a direct-to-vat starter has been amply vindicated.

If two further features are added to the credit side, namely that these ICF cultures are both inexpensive and available for use without any prior preparation, then it is clear that all sectors of the cheese industry are going to examine them with considerable interest:-

The small producer who may welcome their sheer convenience;

The medium-sized operator who may be saved the necessity of rest-day working for starter preparation;

The major manufacturer faced not only with the ever increasing costs of bulk starter production, but also perhaps, with the need to replace a starter facility of declining efficiency;

And how these 'new arrivals' on the 'starter scene' will fare under this intense scrutiny remains to be seen. The ability of the cultures to produce textured cheeses other than Cheddar will also be questioned, but whatever the final outcome, there can be no doubt that these new starters from Eurozyme represent a fascinating advance in starter technology.

Acknowledgement

The author would like to thank Miss Helen Chapman for her most generous assistance in the preparation of this paper, and for making available the results of the production trails carried out in Somerset.

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FERMENTED MILKS

Contents
Yoghurt

YOGHURT

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Yoghurt and similar fermented milks have been produced in the warmer regions around the Mediterranean for centuries, but the more widespread popularity of the product throughout Europe, North America and elsewhere is comparatively recent. These latter markets are dominated by two types of retail product. One variant has a firm, gel-like structure together with a clean, mildly acidic and slightly aromatic flavour – *natural set yoghurt*, while the other has the consistency of 'double cream', and the background flavour of yoghurt is usually modified by the addition of fruit/flavours and sugar (sucrose) – *stirred yoghurt*. Nevertheless, despite the apparently contrasting nature of the end products, the manufacturing procedures for both variants have much in common.

Method of manufacture

The raw material for yoghurt production is usually fresh cow's milk, although the milk from other mammals, such as the sheep, camel or buffalo, is equally amenable to fermentation. Goat's milk can also be employed, but, due to the low level of α_{s1}-casein, the coagulum formed during the fermentation stage is soft and the end product may lack the attractive 'mouth feel' of normal yoghurt. Although the content of milk fat in yoghurt can range from 0.1–4.5% according to taste and/or market demand, the critical feature of the milk, in the present context, is the level of solids-non-fat (SNF). In cow's milk, the level is 8.5–9.0%, of which around 4.5% is lactose, 3.3% protein (2.6% casein and 0.7% whey proteins) and 0.7% mineral salts. Each of these components is vital for the production of a satisfactory yoghurt, in that while the lactose provides an energy source for the starter bacteria (see later), the protein, together with minerals like calcium and phosphorus, gives rise to the basic structure of the gel. However, the levels of protein present in liquid milk are not sufficient to produce a satisfactory end product, and hence the first step in manufacture is to raise the SNF content (see Table 1).

Once the desired level of SNF has been achieved, the milk will be homogenized, often regarded as an optional step, and heat treated. This latter stage, at temperatures well above normal pasteurization, involves passing the milk through a plate heat-exchanger with a holding tube of sufficient capacity to raise the temperature to 90–95°C for 5–10 min.,

Table 1 Outline of the important stages in the manufacture of natural set or stirred fruit yoghurt

Processes	*Materials*	*Comments*
Standardization of fat, addition of skim-milk powder or vacuum/membrane concentration	Full-cream or skimmed milk at 14–16% Solids-Non-Fat, and milk fat (0.1–4.5%)	Sucrose (7–10%) and stabilizers may be added to the base for stirred, fruit yoghurt
Homogenization at 13.79 MPa and 50–55°C	Process milk	Reduces fat globules to <2.0 μm and improves texture of end product
Heat treatment at 80–85°C for 30 min. or 90–95°C for 5–10 min.	Process milk	Reduces bacterial load and oxygen content of milk; denatures whey proteins which interact with *k*-casein and improve texture of end product
Cooling to 30°C or 42°C and inoculation with culture (see text)	Inoculated milk	Set yoghurt will be packaged at this point
Incubation for 16 h at 27–30°C or 3.5–4.5 h at 42°C	Milk coagulated by lactic acid, and flavour/texture compounds released by culture	Incubation rooms for set yoghurt or in-tank incubation for stirred yoghurt
Cooling to 2–4°C for set yoghurt in cartons, 15–20°C for stirred yoghurt in tanks	Fruit purée (10–15% addition rate) or fruit flavours for stirred varieties	The coagulum must be handled carefully to avoid damage to the warm gel
Packaging of stirred, fruit yoghurt and cooling to 2–4°C	Retail products – natural set yoghurt and stirred, fruit yoghurt	120–150 g individual cartons or 500 g family packs are normal

or heating the milk in a process vessel to 80–85°C with a holding time of 30 min. The choice of treatment is simply a reflection of the sophistication of the available plant, but the step is essential to give a yoghurt with the desired textural properties. In particular, heating/holding alters the physicochemical properties of the caseins and denatures the whey proteins. As a result, β-lactoglobulin may become attached to the *k*-casein, so improving the texture (set yoghurt) or viscosity (stirred yoghurt) of the final product, while a partial breakdown of other whey proteins gives rise to products that stimulate the activity of the starter culture.

Microbiology of the process

Once the heat treatment has been completed, the milk is cooled to 42°C prior to inoculation with a culture composed of equal numbers of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (usually known as *Lb. bulgaricus*). A prolonged period of incubation at 27–30°C can be employed if an overnight fermentation is preferred but, as mesophilic contaminants can grow readily at the same temperature, most companies opt for 42°C. The technology for handling the cultures can vary (*See also:* **Starter Cultures**) but, whatever the means of inoculation, it is essential that both species are present (see Figure 1); indeed, retail cartons should, by convention or by law, only be labelled as 'yoghurt' if *Lb. bulgaricus* has been used for the fermentation. Terms such as 'Mild Yoghurt' or 'Bio-yoghurt' are permitted in some countries to describe a product that is 'yoghurt-like' in texture, but the prefix implies that the culture may be different from normal yoghurt.

Fig. 1 A scanning electron micrograph of natural yoghurt showing the long chains of cocci (*Str. thermophilus*) alongside shorter chains of rods (*Lb. bulgaricus*). The matrix of coagulated milk proteins is also clearly visible. (Courtesy of Bottazzi-Bianchi, Institute of Microbiology, UCSC, Piacenza, Italy.)

Str. thermophilus is a Gram-positive bacterium with spherical/ovoid cells of 0.7–0.9 μm diameter (*See also:* **Streptococcus**) and (*See also:* **Structure of Bacteria**), and it is a natural inhabitant of raw milk in many parts of the world. It occurs in milk in long chains of 10–20 cells, and ferments lactose homofermentatively (*See also:* **Metabolism of Bacteria**) to give L(+) lactic acid as the principal product; glucose, fructose and mannose can also be metabolized, but the fermentation of galactose, maltose and sucrose is strain specific. Thus, although the taxonomic characteristics of both *Str. thermophilus* and *Lb. bulgaricus* are well established, numerous strains of each species have evolved, or been selected in the laboratory, and hence the loss or gain of alleles for specific aspects of metabolic performance is not uncommon.

The principal sugar in the yoghurt base, lactose, is actively transported across the cell membrane of *Str. thermophilus* through the mediation of a membrane-located enzyme, galactoside permease, and once inside the cell, the enzyme, β-galactosidase, hydrolyses the sugar to glucose and galactose. The glucose is metabolized to pyruvate via the Embden Meyerhof Pathway (EMP), and lactic dehydrogenase converts the pyruvate to lactic acid. In most strains of *Str. thermophilus*, the galactose and lactic acid leave the cell and accumulate in the medium, but some strains possess a galactokinase that converts the galactose to galactose-1-P, and thence via the Leloir pathway to glucose-1-P which is metabolized in the usual manner. Other strains have the ability to form galactose-6-P which may then be transformed via the tagatose-6-P pathway to glyceraldehyde-3-P and onto pyruvate; the evidence for this latter pathway in *Str. thermophilus* is disputed by some authorities. However, the accumulation of galactose in yoghurt suggests that pathways other than EMP are suppressed in the presence of glucose derived from the hydrolysis of lactose. This latter view is supported by the fact that the uptake of galactose, involving a specific galactose permease, and its subsequent metabolism can only be induced under conditions of lactose deficiency. Indeed, lactose is always the preferred substrate, and even glucose or fructose are absorbed slowly in comparison.

Despite its protein-rich habitat, *Str. thermophilus* displays limited proteolytic ability, and hence its source of nitrogen is, at least initially, free amino

acids occurring naturally in the milk or released during the heat treatment. However, particular free amino acids, such as glutamic acid, histidine, cysteine, methionine, valine or leucine, are not present in milk at levels sufficient to support extensive growth of *Str. thermophilus*, so that the maximum increase in cell numbers is only possible through the absorption of short-chain peptides released by *Lb. bulgaricus* and the hydrolysis of these to the constituent amino acids.

Even though its optimum temperature for growth is 37°C, it can be important that certain metabolic pathways found in *Str. thermophilus*, such as polysaccharide production or the synthesis of acetaldehyde, may operate at different optima. Growth of *Str. thermophilus* ceases at 10°C.

Lb. bulgaricus is also Gram-positive, but occurs in milk as chains of 3–4 short rods with rounded ends, 0.5–0.8 × 2.0–9.0 μm. Its basic metabolism is again homofermentative to give D(–) lactic acid to a level of 1.7–2.1% in milk; this acid tolerance contrasts with that of *Str. thermophilus* which is normally inhibited above 1.0% lactic acid (in the range of pH 4.3–4.5). *Lb. bulgaricus* can, like *Str. thermophilus*, utilize lactose, fructose, glucose and, in some strains, galactose but, unlike *Str. thermophilus, Lb. bulgaricus* can hydrolyse casein, especially β-casein, by means of a wall-bound proteinase to release polypeptides. However, the peptidase activity of *Lb. bulgaricus* is limited and, as *Str. thermophilus* can readily hydrolyse the peptides to free amino acids, it is possible that some of the latter may be used by *Lb. bulgaricus*. The optimum growth temperature for *Lb. bulgaricus* is 45°C, and hence the value of 42°C selected for commercial production is an effective compromise between the growth optima of the two species.

If the yoghurt milk is examined microscopically immediately after inoculation, the balance between the two genera is usually 1 chain of *Str. thermophilus*: 1 chain of *Lb. bulgaricus*, but a differential colony count shows that *Str. thermophilus* accounts for some 75–85% of the colony-forming units (cfu). This contrast reflects the difference in chain length, and it implies that the initial stages of the fermentation are dominated by *Str. thermophilus*.

In part, the use of these two organisms is historical in origin, in that they have frequently been isolated from natural yoghurt made by the indigenous tribes of the Middle East where the high ambient temperature has lead to the selection of thermophilic microfloras in fermented dairy products. Nevertheless, there are good reasons for continuing with the tradition, for when growing in milk, the two organisms interact synergistically. This proto-cooperation is based upon the fact that:

(1) *Str. thermophilus* grows more rapidly than *Lb. bulgaricus*, and releases lactic acid, carbon dioxide from the breakdown of urea in the milk by urease and, usually, formic acid (up to 40 μg ml^{-1}), all of which stimulate the growth and metabolism of *Lb. bulgaricus*; and

(2) *Lb. bulgaricus* hydrolyses some of the casein, and peptidase activity, predominantly originating from *Str. thermophilus*, makes available amino acids that are essential for further development of both species.

The end result of this proto-cooperation is that:

(1) both species actively metabolize lactose to lactic acid, so that the fermentation is complete within 3–4 h (the same number of cells of one species along might take 12–16 h to produce the same level of acidity);

(2) the metabolites liberated by the two species give yoghurt a flavour that is distinctly different from any other fermented milk. Acetaldehyde at levels up to 40 mg kg^{-1} is the main component of the flavour profile, and the major pathway for production by *Lb. bulgaricus*, and to a lesser extent *Str. thermophilus*, is the conversion of threonine to glycine by threonine aldolase.

In the absence of alcohol dehydrogenase in either *Str. thermophilus* or *Lb. bulgaricus*, the acetaldehyde accumulates to a level dependent upon the strains involved; other metabolic pathways, such as the transformation of pyruvate by α-carboxylase, may also be involved with a build-up of acetaldehyde. The rate of formation by *Str. thermophilus* is temperature dependent, and the activity of threonine aldolase decreases significantly as the temperature of incubation is raised above 30°C. The comparable enzyme in *Lb. bulgaricus* is unaffected by temperature, and hence this latter organism is probably the main source of acetaldehyde in commercial yoghurt. Other compounds of starter origin, such as free fatty acids, amino acids, acetone, diacetyl and keto/hydroxy acids, contribute to the final flavour, but the importance of such materials with respect to perceived taste and aroma is poorly understood; and

(3) some strains of the two species can produce appreciable levels of extracellular, polysaccharide materials, such as glucans or polymers involving glucose, galactose and rhamnose as the constituent sugars. The presence of these metabolites enhances considerably the viscosity/

$$\underset{\text{Threonine}}{CH_3\text{-}CHOH\text{-}CH\text{-}NH_2\text{-}COOH} \rightarrow \underset{\text{Glycine}}{CH\text{-}NH_2\text{-}COOH} + \underset{\text{Acetaldehyde}}{CH_3\text{-}CH{=}O}$$

consumer appeal of the end product, because while some of the polysaccharide forms a layer over the bacterial cells, the remainder forms a network that binds the cells and the casein together as a viscous mass; commercial starter manufacturers have available a range of cultures that differ with respect to polysaccharide synthesis. Thus, strains differ with respect to both the type of polysaccharide formed and its quantity and, in the example shown in Figure 2, *Lb. bulgaricus* synthesizes a 'gum-like' material whilst *Str. thermophilus* produces a 'slimy', 'glucan-like' polymer. The result is that blending the *Lb. bulgaricus* with a nonpolysaccharide-producing yoghurt culture (A) increases the viscosity of the yoghurt in line with rate of addition. By contrast, the low-viscosity polysaccharide from *Str. thermophilus* enhances the viscosity of the yoghurt at low levels but, as the level increases along with the raised inoculation rate (1:1 ratio), the 'fluid' nature of the material means that no improvement in the viscosity of the product is manifest. In addition, other strains of *Str. thermophilus* possess a glycohydrolase capable of hydrolysing polysaccharides, and such activity can also influence the impact of a given culture on viscosity.

Once the milk has been inoculated, it will follow one of the two routes illustrated in Figure 3 and, as the fermentation begins, the population of *Str. thermophilus* develops rapidly until it accounts for over 90% of the bacterial cells present. However, over the next two hours the synergistic influence of the streptococci encourages more rapid growth and metabolism in *Lb. bulgaricus* and, after 4 h, the balance between the populations will be restored to that found in the freshly inoculated milk. A tendency that is strengthened by the fact that the growth of *Str. thermophilus* is progressively inhibited by the accumulation of lactic acid, while the same conditions stimulate the activity of *Lb. bulgaricus*. The end result of these interactions is that when the fermentation is complete, i.e. the acidity of the milk has

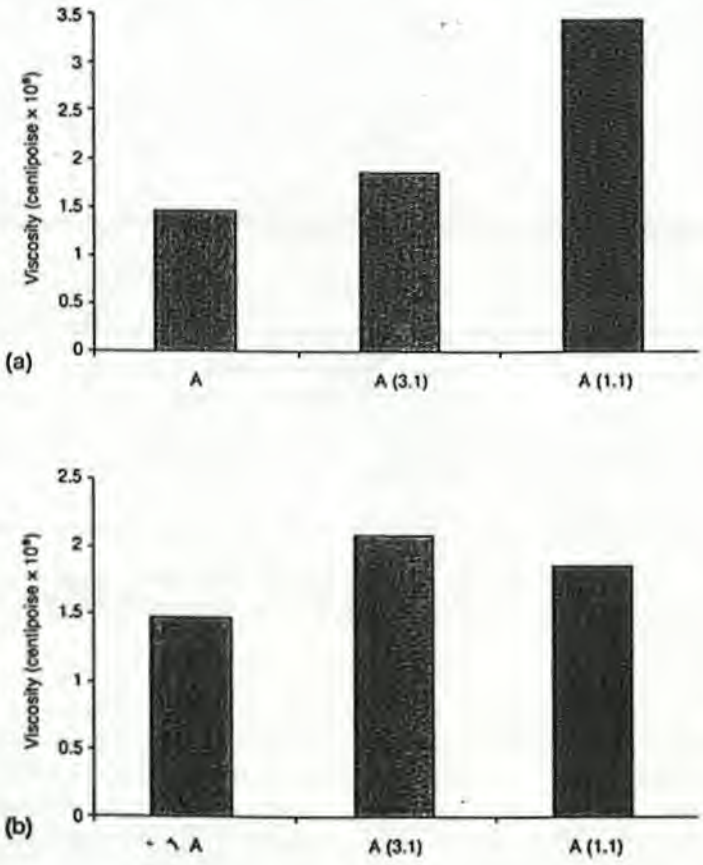

Fig. 2 The effect on the viscosity of yoghurt of adding increasing volumes of either (a) *Lb. bulgaricus* ('gum' producer) or (b) *Str. thermophilus* ('slime' producer) to a standard, nonpolysaccharide-producing starter culture.

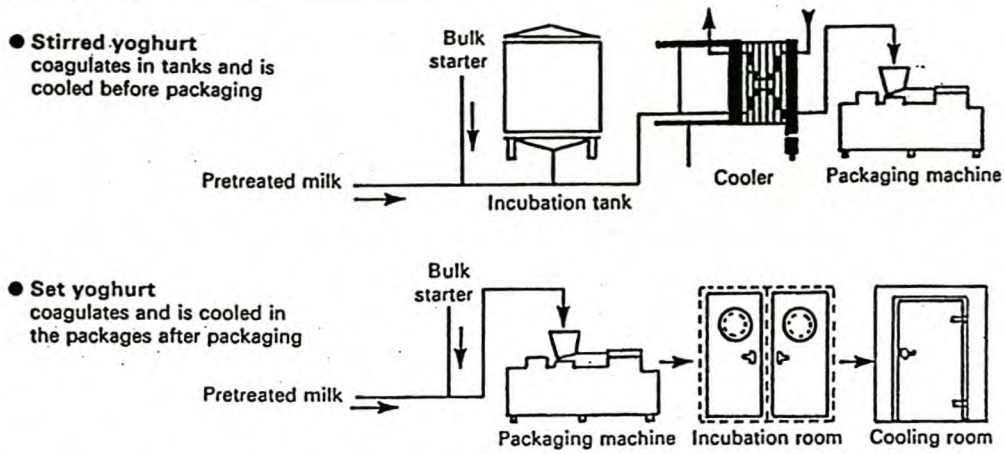

Fig. 3 The principal stages involved in the manufacture of yoghurt.

risen to 1.2–1.4% lactic acid (around pH 4.2–4.3), the populations of each starter organism may well exceed 20×10^6 cells ml^{-1}.

At an acidity of 1.2–1.4% lactic acid, which is probably the level preferred by most consumers, the milk proteins will have coagulated to form a firm gel, and the product must be cooled to avoid over-acidification. If this control is not exercised, then (1) the product may develop an excessively sharp sour taste, and (2) the protein gel may begin to shrink and cause whey to separate as a discrete layer on the surface of the yoghurt. This free whey can, of course, be stirred back into the body of the product, but in set yoghurts, at least, its presence must be regarded as a fault.

Inhibitors of starter activity

The production and/or employment of starter cultures in a yoghurt factory can lead to the microbiological problems detailed below.

Contamination Contamination of the starter culture by adventitious bacteria, yeasts or moulds or, possibly, bacteriophage. Problems from these sources should be containable with proper attention to hygiene (*See also:* HACCP) but, because the risk is always present, many yoghurt manufacturers are turning to direct-to-vat cultures (*See also:* Starter Cultures). These concentrated cultures are, of course, added direct to the process milk, and hence the routes for possible contamination are reduced to a minimum; in addition, although phage have been isolated for both *Str. thermophilus* and *Lb. bulgaricus*, their presence is not as serious as with mesophilic fermentations. This contrast appears to stem from the facts that: (1) cheese milk is stirred continuously during acidification, and hence any phage released by lysis of bacterial cells are distributed throughout the vat; and (2) the undisturbed yoghurt milk coagulates quite rapidly, so that any proliferation of phage remains localized.

Changes in culture activity The activity of the culture may change, e.g. rate of acid production or level of aroma/flavour compounds secreted, as a consequence of routine sub-culturing. Exactly why these latter changes arise is not clear, for whereas the loss of plasmids encoded for characteristics of technological importance, e.g. proteolytic activity, antibiotic resistance or the secretion of glycoproteins, is well documented for the cultivation of *Lactococcus* spp., neither *Lb. bulgaricus* nor *Str. thermophilus* appear to possess any plasmids of metabolic significance.

Presence of antibiotics and other inhibitory substances in the milk *Str. thermophilus* is especially sensitive to antibiotics such as penicillin, streptomycin, neomycin and ampicillin which are widely used to control mastitis and, when grown alone, contamination as low as 0.004 International Units (IU) of penicillin can inhibit cell wall development; strains of *Lb. bulgaricus* tend to be a little more tolerant (0.02 IU of penicillin). However, even when the two organisms are growing together under optimum conditions, 0.01 IU of penicillin can delay fermentation. Sanitizing agents employed to clean a plant, such as chlorine (100 mg l^{-1}) or iodophors (60 mg l^{-1}), can cause inhibition of the mixed cultures, and hence the screening of bulk milk for microbiocidal agents is essential.

Strain incompatibility Incompatibility between strains of *Lb. bulgaricus* and *Str. thermophilus* can result in an almost complete absence of proto-

cooperation between the species, with the result that coagulation times are increased by several hours, and the organoleptic quality of the end product may be extremely poor. In practice, the manufacturers of starter cultures take great care to select and blend only compatible strains, but an attempt by a yoghurt-maker to mix cultures from different sources could lead to poor performance.

Final processing

For yoghurt coagulated in retail cartons, cooling can be achieved by blowing cold air through the incubation room, while in-tank cooling of the base for stirred yoghurt requires the circulation of chilled water (2°C) through the jacket/in-tank cooling system of the vessel, or pumping the warm yoghurt (42°C) through a plate or tubular cooler; in the latter case, great care is necessary to avoid physical damage to the coagulum.

Once the yoghurt has been cooled to around 20°C, the metabolic activity of the culture almost ceases, and the product can be handled as described in Table 1.

Considerations of microbiological quality

The severe heat treatment received by process milk, together with the low pH of the final product, make yoghurt extremely safe in respect to public health, because none of the recognized pathogens can survive or grow below pH 4.3. For example, the spores of *Bacillus cereus* will not germinate at low pH, while organisms of concern in soft cheeses, e.g. *Listeria monocytogenes*, will be inactivated long before the yoghurt reaches the consumer. In addition, there is good evidence that metabolites from the yoghurt organisms can actively depress the viability of many enteric pathogens, such as *Campylobacter*, *Escherichia* or *Salmonella* spp. Hydrogen peroxide is one such metabolite released by *Lb. bulgaricus*, and some strains of the same organism are reported to secrete an antibiotic called bulgarican; *Str. thermophilus* may also release a low-molecular-weight compound with bacteriocidal properties. However, the activity of these bacteriocins is strictly limited and, in reality, they do little more than reinforce the effect of acidity.

Spoilage, however, can occur through the activities of acid-tolerant yeasts, or occasionally moulds, and widely distributed yeasts, like *Candida* or *Saccharomyces* spp., can be associated with gas formation and/or carton 'doming' of fruit yoghurts. In this situation, the natural fruit sugars provide an abundance of substrates for fermentation but, as 'doming' indicates a yeast count in the region of 10×10^4 cfu g^{-1} of yoghurt, it is likely that the source of the original contamination was either a 'dirty' plant or unpasteurized fruit; in the absence of severe temperature abuse, e.g. storing the yoghurt at 10–15°C, casual air-borne yeasts rarely proliferate sufficiently to cause problems. In natural yoghurt, lactose is the principal sugar available and, as few yeasts can ferment lactose, the major concern is species like *Kluyveromyces marxianus* var. *lactis* or *K. marxianus* var. *marxianus*. Both of these lactose-utilizing species grow readily on poorly-cleaned surfaces in a dairy plant, and hence high standards of hygiene are essential if post-heat treatment contamination is not to occur.

In cold Northern climates, cases of yeast spoilage should be infrequent but, even so, it is generally recommended that yoghurt for the retail market should have a yeast count of <10 cfu g^{-1} (*See also:* Colony Counts). The same figure would apply also for moulds, for genera such as *Mucor*, *Rhizopus*, *Penicillium* or *Aspergillus* can grow readily at the yoghurt/air interface of an undisturbed carton. Equally important is the fact that, unlike the situation with yeasts, just one spore of a fungus can spoil a carton by growing over the surface of the product, and hence protection of a filling-line from air-borne contamination is essential.

In warmer regions where active spoilage might be expected, the solution is either to reduce the 'sell-by' date of the product from 2–3 weeks (typical of the UK) to 4–5 days, or if regulations permit, add sorbic acid (usually as potassium sorbate) at a level up to 300 mg kg^{-1}. This preservative is extremely effective against yeasts, but has little effect on *Lb. bulgaricus* or *Str. thermophilus*. This latter point is important, as yoghurt, by definition, must contain an 'abundant population of viable bacteria of starter origin'; if any processing technique interferes with this population, then the consumer has the right to be informed on the packaging; for example, yoghurt that has been subject to heat treatment after fermentation must be designated as 'heat treated' or 'pasteurized' yoghurt.

Excessive acidity, as a result of continued starter activity during prolonged storage above 5°C, for example, can also be a problem, because the acid-tolerant *Lb. bulgaricus* has the ability to generate lactic acid to levels of 1.7% or even above – depending upon the strain. Such a level is too harsh for the palates of most consumers, and it is this post-production acidification that, in general, tends to determine the shelf-life of commercial yoghurt. Some protection against the increase in lactic acid can be obtained by lowering the level of *Lb. bulgaricus* in the original inoculum but, as this practice can reduce the degree of synergism, such products tend to lack the characteristic flavour of 'yoghurt'.

Table 2 Some typical water-soluble vitamin contents of skim-milk and low-fat yoghurt; variations between the two products tend to result from synthesis or utilization by the starter culture

Vitamin	*Milk*	*Yoghurt*
Thiamin (B_1)	42	40
Riboflavin (B_2)	180	200
Pyridoxine (B_6)	42	46
Cobalamin (B_{12})	0.4	0.2
Folic acid	0.3	4.1
Nicotinic acid	480	125
Pantothenic acid	370	380
Biotin	1.6	2.6
Choline (mg)	4.8	0.6

Units as $\mu g\ 100\ g^{-1}$ except as indicated.

Nutritional considerations

The increase in SNF will, of course, improve the compositional analysis of yoghurt versus milk, e.g. 5.0% total protein versus 3.4% in milk or 180 mg ml^{-1} of calcium versus 120 mg ml^{-1} in milk and, in addition, the availability of these nutrients is enhanced as a result of the fermentation. For example, the digestion of the milk protein is much more rapid than when the same quantity is consumed in liquid milk, while the acid nature of the product also alters the bioavailability of minerals like calcium and zinc. Some changes in the levels of water-soluble vitamins have been recorded following fermentation (see Table 2), but the extent of any increase or decrease is rather variable; a pattern that reflects differences between the strains of *Lb. bulgaricus* and *Str. thermophilus* comprising the starter cultures available on the market.

As mentioned earlier, all yoghurts should contain high counts of starter bacteria, and most authorities believe that their presence is beneficial to the consumer. The fact that no strains of *Str. thermophilus* are able to survive the low pH of the stomach (around pH 2.0), or the action of bile salts in the small intestine, is well known, and few strains of *Lb. bulgaricus* fare any better. Nevertheless, as these stresses cause the starter bacteria to autolyse, so lactase (β-galactosidase) is released into the lumen of the intestine, with the result that most of the residual lactose from the yoghurt is hydrolysed before the intestinal contents reach the colon. This action enables lactose maldigestors (adults who cannot drink milk because the illeum has lost the facility to secrete lactase) to consume yoghurt with ease, whereas the same quantity of milk would give rise to severe abdominal discomfort as the bacteria in the colon ferment the lactose with the release of carbon dioxide. In addition, there is evidence that the regular ingestion of yoghurt starter bacteria assists with the maintenance of healthy populations of lactobacilli in the distal region of the small intestine and bifidobacteria in the colon. The precise mechanism for this stimulation is not clear, but it seems likely that vitamins or other growth factors released from the degenerating cells of the starter culture have this desirable impact.

It is relevant also that *Str. thermophilus* produces the L(+) isomer of lactic acid, for this form is completely metabolized by the body with the release of energy. Ingestion of the D(−) isomer secreted by *Lb. bulgaricus* can, especially in young children, give rise to metabolic disturbances but, as early acidification is dominated by the activity of *Str. thermophilus*, the lactic acid in normal yoghurt is mainly the L(+) isomer.

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Enumeration of starter cultures in fermented milks

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Summary. Some media available for the isolation and enumeration of starter cultures employed for the manufacture of cheese, yogurt and bio-yogurt were examined. Reddy's medium or a modification of Elliker's medium was found to be most satisfactory for *Lactococcus* spp., while trypticase phytone yeast (TPY) agar with a mixture of antibiotics proved suitable for the discrete enumeration of *Bifidobacterium* spp. The inclusion of Prussian blue (PB) in reinforced clostridial medium or tryptone proteose peptone yeast extract (TPPY) agar gave excellent differential counts for the starter bacteria in yogurt even when the culture was imbalanced, while TPPY (PB) agar allowed the visible separation of all four of the organisms that might be found in a typical bio-yogurt, namely *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, a *Bifidobacterium* sp. and *Lb. acidophilus*. It was noted that variation among different strains of any given species could change the expected reactions, so for quality control purposes the suggested media may need to be modified to cope with the specific cultures in use.

Many dairy-related projects include an assessment of the survival of starter cultures in a retail product. Yogurt or cheese cultures are the most widely used in the dairy industry, but *Bifidobacterium* spp. and *Lactobacillus acidophilus* are increasingly to be found in bio-yogurt and similar products. The essential interest of a programme may be the total colony count of bifidobacteria or *Lb. acidophilus* with respect to achieving a 'therapeutic minimum' (Robinson, 1989) or, where it is considered that product quality may be affected by an imbalance between the constituent organisms, the relative proportions of individual groups could be the priority. The approach to such evaluations tends to involve (a) the selective isolation of just one genus, e.g. during the enumeration of bifidobacteria in a dairy product, (b) the selective isolation of one genus, e.g. *Lactococcus*, but with differentiation between species or subspecies of that same genus or (c) selective isolation and differentiation of disparate genera, e.g. during the examination of a yogurt or bio-yogurt.

The final choice of method may have to be modified to take account of the foodstuff, the species or strains of the organisms to be isolated and the nature of any competing genera, and hence it is not surprising that many different options have been developed. Nevertheless, the growing trend of blending a number of genera into one starter culture, e.g. bifidobacteria and other yogurt culture genera, has raised serious problems in routine quality control. Procedures that necessitate the enumeration of three or four different microorganisms on separate media involve much time and expense, but complete differentiation of the expected cultures on a

single medium may often prove unattainable, particularly if the product includes casual contaminants as well.

However, a protocol using two media at any one time might be realistic, and hence it was decided to examine the difficulties in separating, isolating and enumerating from test samples of fermented milk (a) a yogurt starter culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) present alone or in association with a *Bifidobacterium* sp. and *Lb. acidophilus*, (b) a standard cheese culture containing *Lactococcus lactis* subsp. *lactis* (*Lc. lactis*) and *Lc. lactis* subsp. *cremoris* (*Lc. cremoris*) alone or in the presence of a *Bifidobacterium* sp. and (c) a *Bifidobacterium* sp. alone or in the presence of a starter culture for yogurt or cheese.

MATERIALS AND METHODS

Cultures

Individual freeze-dried cultures were obtained from the National Collection of Food Bacteria (NCFB (formerly known as the National Collection of Dairy Organisms, NCDO), Institute of Food Research, Reading Laboratory, Reading RG6 6BZ) and the National Collections of Industrial and Marine Bacteria (NCIMB, Aberdeen AB2 1RY).

The specific strains examined were *Str. thermophilus* NCDO 1469, *Lb. bulgaricus* NCFB 1489, *Lb. acidophilus* NCDO 1693, *Lc. lactis* NCDO 763, 1007 and 276, *Lc. cremoris* NCDO 607 and NCIMB 8662, *Bifid. bifidum* NCFB 2715 and *Bifid. adolescentis* NCFB 2204. A standard, freeze-dried starter culture for yogurt (CH1, Chr. Hansen's Laboratory, Reading RG2 0QL) was also examined. Each culture was propagated in sterilized (121 °C for 2 min) reconstituted skim milk (120 g total solids/l). After incubation at the optimum temperatures for the organisms (see Tables 1 and 2) until the pH values had fallen to ~ 5·0, an inoculum (50 ml/l) from each culture was transferred to freshly prepared skim milk and incubated to coagulation point. The cultures were then stored at 5 °C and subcultured each week.

Enumeration of the test cultures growing in milk

Enumeration of Str. thermophilus *and* Lb. bulgaricus. Culture media for the enumeration of starter bacteria in yogurt can be divided into three: (a) general media that will give an overall total colony count without differentiating between *Str. thermophilus* and *Lb. bulgaricus*, e.g. Elliker's medium (Elliker *et al.* 1956), (b) media formulated to isolate selectively either *Str. thermophilus* or *Lb. bulgaricus*, e.g. acidified MRS medium (De Man *et al.* 1960) for isolating *Lb. bulgaricus*, or M17 (Terzaghi & Sandine, 1975; Shankar & Davies, 1977) for *Str. thermophilus* and (c) differentiating media that permit the enumeration of *Str. thermophilus* and *Lb. bulgaricus* as separate, and visually identifiable, colonies on the same plate.

This last approach offers a laboratory a number of advantages, so we examined the performance of five differentiating media: Lee's medium (Lee *et al.* 1974), L–S differential agar (Eloy & Lacrosse, 1976), tryptose proteose peptone yeast extract–eriochrome (TPPY) agar (Bracquart, 1981), modified lactic agar (Matalon & Sandine, 1986) and reinforced clostridial Prussian blue (RCPB) agar (Onggo & Fleet, 1993). In addition, M17 and acidified MRS, as recommended by International Dairy Federation (1988), were used as a further check on the numbers of streptococci and lactobacilli present in the relevant dilutions. Elliker's agar was included to give an overall total count. The conditions of incubation are given in Table 1, but aerobic incubation of all media at 42 °C was included for comparison. If complete

differentiation of *Str. thermophilus* and *Lb. bulgaricus* were possible under aerobic conditions, then the medium might well allow the isolation of yogurt bacteria in the presence of anaerobic bifidobacteria.

For evaluation of the selected media, culture CH1 (10 ml) was added aseptically to sterile peptone (90 ml, 1 g/l), and serial dilutions (1 ml into 9 ml of diluent) were prepared down to 10^{-5}–10^{-7}. The same procedure was applied to the individual cultures of *Str. thermophilus* and *Lb. bulgaricus*, which were then plated separately on to all the differential media so that recovery could be compared against M17 and acidified MRS. This also allowed confirmation of the expected reactions.

Before plating out the selected dilutions, the sterilized media were poured into petri dishes, and the surfaces of the solidified agars were dried under a laminar flow hood for ~ 30 min. Portions (0·1 ml of the appropriate dilutions (10^{-5}–10^{-7}) were then pipetted on to duplicate plates at each dilution and spread using a sterile bent glass rod. Incubation was carried out at the recommended temperature and under aerobic or anaerobic conditions (see Table 1). Anaerobic conditions were obtained using polycarbonate jars enclosing a gas generating kit (Oxoid Ltd, Basingstoke RG24 0PW) capable of liberating ~ 1·8 l hydrogen and 350 ml carbon dioxide.

Apart from the assurance provided by the examination of the individual cultures of *Str. thermophilus* and *Lb. bulgaricus* on the selective agars, a random sample of colonies from the yogurt culture was checked, following differentiation, for the presence of cocci or rods using an Olympus KHC light microscope (Olympus Optical, London EC1Y 0TX). The Gram staining procedure described in Harrigan & McCance (1976) was used.

Enumeration of Lc. lactis *and* Lc. cremoris. The culture media for *Lactococcus* spp. can be divided into two: general and differential. As the relative proportions of the two subspecies of *Lc. lactis* can be important in cheesemaking, selective enumeration on Turner's medium (Turner *et al.* 1963) or Reddy's medium (Reddy *et al.* 1969) is often preferred. The performance of these two media was therefore examined in detail, as was their ability to select against bifidobacteria.

For enumeration of the cheese starter bacteria, batches of Turner's medium and Reddy's medium were poured into petri dishes, and spread plates were prepared from serial dilutions of two composite cultures consisting of equal volumes of (a) *Lc. lactis* NCDO 1007 and *Lc. cremoris* NCDO 607 and (b) *Lc. lactis* NCDO 276 and *Lc. cremoris* NCIMB 8662. The temperatures of incubation were 30 °C for Turner's medium and 32 °C for Reddy's medium, and anaerobic conditions were maintained as for the yogurt cultures (see above).

In order to confirm the differentiation recorded on the media, a random selection of colonies from each group was tested for sugar fermentation and arginine hydrolysis. Only *Lc. lactis* can ferment ribose, maltose, trehalose and aesculin (Thompson & Collins, 1989), and it also produces ammonia from arginine in Møller's decarboxylase medium (Møller, 1955).

Differences in the fermentation profiles of *Lc. lactis* and *Lc. cremoris* were monitored by adding a filter-sterilized solution (5 ml, 100 g/l) of lactose, maltose, ribose, trehalose or aesculin to 100 ml Elliker's agar (excluding any carbohydrate and sterilized at 121 °C for 15 min). Bromocresol purple (2 ml, 1 g/l) was added aseptically, and the complete medium (10 ml portions) was dispensed into screwcap tubes. Once the agar had solidified, 0·1 ml of a suspension of *Lc. lactis* or *Lc. cremoris* in M17 broth was stabbed into the surface of each slope. These tubes were incubated at 30 °C. and compared with the controls devoid of sugar. The production of a yellow zone around the inoculum within 48 h was taken as a positive result.

Enumeration of Bifidobacterium *spp.* The choice of medium for enumerating bifidobacteria depends on the type of product under investigation. If the material contains bifidobacteria alone, then a nutrient-rich, non-selective medium, such as blood liver agar (Mitsuoka *et al.* 1965), modified Rogosa agar (Shimada *et al.* 1977) or trypticase phytone yeast (TPY) agar (Scardovi, 1986) can be used. However, in the case of products containing bifidobacteria together with yogurt or cheese starters, a selective medium becomes essential (Rašić, 1990). To this end, various antimicrobial substances have been added to media to prevent the growth of all lactic acid bacteria except bifidobacteria, and sodium propionate and lithium chloride have been proposed (Lapierre *et al.* 1992). The selective action of several types of antibiotic, alone or in mixtures, such as paramomycin sulphate, neomycin sulphate and nalidixic acid, has been reported by many workers (Ochi *et al.* 1964; Mitsuoka *et al.* 1965; Shimada *et al.* 1977; Teraguchi *et al.* 1978).

On the basis of the published information (Samona & Robinson, 1991), blood liver agar, modified Rogosa agar and TPY agar were examined, together with the following solutions of antibiotic mixtures. These contained (mg/l) NPNL: neomycin sulphate 100, paramomycin sulphate 200, nalidixic acid 15, lithium chloride 3000; PPNL: neomycin sulphate 200, paramomycin sulphate 50, sodium propionate 15000, lithium chloride 3000; LP: lithium chloride 2000, sodium propionate 3000. Each composite solution was added to the basic medium at a rate of 50 ml/l except for one trial with NPNL at 20 ml/l.

Pure cultures of *Bifid. bifidum* and *Bifid. adolescentis* were surface-plated on to blood liver agar, modified Rogosa agar and TPY agar as 0·1 ml volumes of 10^{-5}–10^{-7} dilutions (duplicate plates at each dilution). The same dilutions were used on the selective media (see Table 2), but in these cases 0·1 ml from a dilution (10^{-2}–10^{-4}) of either the yogurt culture (CH1) or a 50:50 mixture of *Lc. lactis* and *Lc. cremoris* was added at the same time. Modified Chalmer's agar (Vanos & Cox, 1986), which was reported to be selective for all lactic acid bacteria except bifidobacteria, was employed as directed by the authors. Plates inoculated with bifidobacteria alone, or in combination with the yogurt culture, were incubated at 37 °C for 72 h, while plates co-inoculated with the cheese culture were held at 30 °C for 48 h. Anaerobic conditions were applied throughout.

Each of the media employed was prepared in the laboratory from the individual ingredients.

RESULTS AND DISCUSSION

Differentiation of yogurt culture

As can be seen from Table 1, Lee's medium gave excellent recovery and differentiation (*Str. thermophilus*, yellow colonies; *Lb. bulgaricus*, white colonies) with both combined and pure cultures. However, in a parallel trial with commercially produced yogurt in which the strains of starter bacteria were not known, the performance of the medium tended to be erratic. Lee *et al.* (1974) acknowledged this problem, and suggested that varying activity of *Lb. bulgaricus*, with respect to rates of release of lactic acid, was responsible. It was decided therefore that TPPY agar (*Str. thermophilus* round white–violet colonies; *Lb. bulgaricus*, irregular clear colonies) or RCPB agar (*Str. thermophilus*, small pale blue colonies with a thin light blue zone in the medium; *Lb. bulgaricus*, large pale blue colonies with wide royal blue zone in the medium) may be better for the routine enumeration of the yogurt bacteria. On both of these media, recovery and differentiation (confirmed by Gram staining of selected colonies) were as good as on Lee's medium.

Table 1. *Relative performance of some media employed to enumerate the species in a standard yogurt culture, together with single strain, individual cultures of* Lactobacillus delbrueckii *subsp.* bulgaricus (*NCFB 1489*) *and* Streptococcus thermophilus (*NCDO 1469*)†

(Values for counts are given as cfu ($\times 10^{-6}$)/ml culture)

	Incubation			Differential count§		Pure cultures	
Medium‡	Temperature, °C	Time, h	Conditions	*Lactobacillus*	*Streptococcus*	*Lactobacillus*	*Streptococcus*
Lee's medium	37	48	Anaerobic	1300	5000	530	1120
TPPY agar	42	24	Anaerobic	300	2700	680	1190
TPPYPB agar	37	48	Anaerobic	200	3000	800	1180
Modified lactic agar	37	48	Anaerobic	200	1200	21	106
L-S differential agar	42	48	Aerobic	(48)§		14	65
RCPB agar	37	48	Anaerobic	500	3200	770	1130
Elliker's agar	37	48	Anaerobic	(900)		500	1010
M17	37	48	Aerobic	—	560	—	1140
Acidified MRS	37	72	Anaerobic	105	—	660	—

--, No growth was observed at dilutions used (10^{-6}–10^{-8}).

† Each culture was examined (duplicate plates) on all the media; the values are overall means from two separate trials with the cultures grown under identical conditions. For details, see text. There were no significant differences between trials.

‡ For full names of and references to the media, see text.

§ Values in parentheses indicate that no differentiation was observed.

Modified lactic agar and L–S differential agar both failed to provide reliable differentiation, and microscopic examination was needed to confirm the identity of any given colony. The reason(s) for this poor performance were not determined. None of the selected media gave good recovery or differentiation when the temperature was raised to 42 °C and the conditions rendered aerobic, so that this combination could not be used to select against bifidobacteria. Acidified MRS agar, M17 agar and Elliker's medium performed as expected (see Table 1), and the results served to confirm that the recoveries on the differential media were of the correct order of magnitude.

However, in order to check whether bifidobacteria could be counted on any of the media alongside the yogurt culture, equal volumes (10^{-5}–10^{-7} dilutions) of the yogurt cultures and either *Bifid. bifidum* or *Bifid. adolescentis* were mixed. Portions (0·1 ml) were then plated out as before and on RCPB agar *Bifid. bifidum* and *Bifid. adolescentis* appeared as white colonies, while *Lb. bulgaricus* and *Str. thermophilus* emerged as pale blue colonies surrounded by wide royal blue or thin light blue zones respectively. Therefore, in the mixed cultures of yogurt bacteria and *Bifid. bifidum* or *Bifid. adolescentis*, the three genera could be easily distinguished.

The addition of Prussian blue to TPPY agar (TPPYPB) gave even more clear-cut results than those obtained with RCPB agar and, when the inoculum was modified to include the yogurt culture, bifidobacteria and a similar cell count of *Lb. acidophilus*, all four organisms could be enumerated on the one medium. Colonies of *Bifidobacterium* sp. and *Str. thermophilus* appeared the same as on RCPB agar, *Lb. bulgaricus* produced small shiny white colonies surrounded by a wide royal blue zone, and *Lb. acidophilus* produced large pale blue colonies surrounded by a wide royal blue zone. Differences between strains might prove relevant, but if a range of commercial cultures performed similarly TPPYPB agar could provide a convenient medium for the routine examination of bio-yogurt fermented with mixed cultures of 'health-promoting' and other bacteria.

Differentiation of Lc. lactis *and* Lc. cremoris

Although the recovery of *Lactococcus* spp. on both Reddy's medium and Turner's medium was equally good in terms of total colony counts, the expected differentiation of *Lc. lactis* (red colonies) and *Lc. cremoris* (cream colonies) on Turner's medium proved extremely difficult to interpret. On Reddy's medium, the separation of the colonies was as expected (*Lc. lactis*, white colonies; *Lc. cremoris*, yellow colonies) but, although the performance of the medium could not be faulted, it had the disadvantage of being extremely time consuming to prepare.

Consequently, an alternative approach based upon that employed by Lee *et al.* (1974) for yogurt bacteria was examined. In this case, modified Elliker's medium (excluding the yeast extract and sugars) was used as the base, and the differentiating sugars were lactose (1 g/l) and maltose (5 g/l). Bromocresol purple was employed as the indicator, and the expectation was that *Lc. lactis* would be able to ferment both sugars but *Lc. cremoris* would be able to utilize only lactose. Thus it was expected that *Lc. lactis* would produce sufficient acid to give rise to colonies with a distinct yellow colouration, while those of *Lc. cremoris* would appear white. In practice, good recovery and differentiation were achieved on this modified medium and, given the ease of preparation, it would appear to offer an acceptable alternative to Reddy's medium. However, like Reddy's medium, it was not selective against bifidobacteria, and hence it could not be employed to monitor starter activity in a cheese containing a probiotic culture as well (Tamime *et al.* 1995).

able 2. *Total colony counts of the organisms indicated on a range of general and selective media that have been recommended for the enumeration of bifidobacteria*†

(Values are given as cfu ($\times 10^{-6}$)/ml culture)

Inoculum dilutions...		10^{-6}–10^{-8}		10^{-2}–10^{-4}	
Medium	Supplement‡	*Bifid. bifidum*	*Bifid. adolescentis*	Yogurt culture	Cheese culture
Blood liver agar	None	510	850	Not tested	
	NPNL	280	0	0	0
	PPNL	0	0	0	0
Modified Rogosa agar	None	202	720	Not tested	
	NPNL	180	0	0	0
	PPNL	0	0	0	0
TPY agar	None	250	790	Not tested	
	NPNL	340	0	0	0
	PPNL	0	0	0	0
	NPNL§	190	680	0	0
	LP	630	1140	$> 10^4$	$> 10^4$

† Each culture was examined (duplicate plates) on all the media; the values are overall means from two separate trials with the cultures grown under identical conditions. Plates were incubated anaerobically at 37 °C for 72 h for bifidobacteria alone or with yogurt culture, or at 30 °C for 48 h for cheese culture. For details, see text. There were no significant differences between trials.

‡ Supplements contained (mg/l) NPNL: neomycin sulphate 100, paramomycin sulphate 200, nalidixic acid 15, lithium chloride 3000; PPNL: neomycin sulphate 200, paramomycin sulphate 50, sodium propionate 15000, lithium chloride 3000; LP: lithium chloride 2000, sodium propionate 3000. Each composite solution was added to the basal medium at 50 ml/l except §, 20 ml/l.

Differentiation of Bifidobacterium *spp.*

As expected, the bifidobacteria grew well on all the media without selective agents (Table 2), but on blood liver agar supplemented with NPNL only *Bifid. bifidum* gave acceptable growth. The same contrast was observed on modified Rogosa agar with added NPNL, in that the mixture of antibiotics at 50 ml/l inhibited the yogurt and cheese cultures as well as *Bifid. adolescentis*. Modified Rogosa agar made selective with PPNL was inhibitory for both species of *Bifidobacterium*. The same pattern was repeated with the selected versions (NPNL and PPNL) of TPY agar. Successful growth of both species of *Bifidobacterium* in the presence of LP was masked by the growth of the starter bacteria.

Since better recovery of *Bifid. bifidum* was observed with NPNL, the use of lower concentrations of NPNL (10–40 ml/l) in TPY agar was tested. It was found that at 20 ml/l NPNL gave good recovery for both *Bifid. bifidum* and *Bifid. adolescentis* while at the same time the growth of cheese and yogurt cultures was inhibited (Table 2). However, when *Lc. lactis* NCDO 763 was substituted for *Lc. lactis* NCDO 276 in a further trial, the cheese culture grew well in the presence of 20 ml NPNL/l. Clearly the concentration of antibiotic will have to be carefully adjusted to suit the precise strains being employed for any given product. Both species of bifidobacteria grew readily on modified Chalmer's agar, but the reason(s) for the contrast with the expected results (Vanos & Cox, 1986) was not examined.

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Acetaldehyde as an indicator of flavour intensity in yogurt

IN assessing the flavour of a foodstuff, the most obvious approach is the organoleptic one. However, although the consumer palate will always remain the ultimate judge, meaningful sensory analysis on an experimental basis can prove difficult and, on a routine basis, often impossible. There is, therefore, every incentive to improve the rôle of instrumental analysis in quality control procedures associated with flavour, and one such approach involves the monitoring of a key component of the relevant chemical profile. It was this latter approach that was selected in relation to yogurt, with "acetaldehyde" acting as the indicator of quality.

Thus, a number of workers have indicated an apparent correlation between the level of acetaldehyde in natural yogurt and intensity of the characteristic flavour (Lindsay & Day, 1965; Bills *et al.*, 1972; Pette & Lolkema, 1950), but there appears to be little conviction that the association could be of practical value. The reasons for this reticence can probably be summarised as follows:

(a) a feeling that flavour components of natural yogurt need not include acetaldehyde (Schulz & Hingst, 1954);
(b) the absence of any definite evidence linking consumer preference with levels of acetaldehyde;
(c) the absence of a simple, reliable method of analysing yogurt for acetaldehyde. (Veringa & Schriver — Davelaar, 1970).

The first point is obviously incapable of resolution, because it is quite possible that a fortuitous combination of starter organisms could produce an acceptable yogurt with little acetaldehyde. However, such a situation would probably be atypical, and hence it was hoped that the employment of commercially available starters would largely nullify this criticism.

It was decided, therefore, to produce a number of yogurts using different starters, with the restriction that they should contain solely *Streptoccus thermophilus* and *Lactobacillus bulgaricus*. This restriction was employed because many commercial starters are of this type, and also because the interaction between the two organisms is reported to enhance the production of acetaldehyde (Hamdan *et al.*, 1971).

The different samples were then subjected to concurrent organoleptic appraisal and analysis for acetaldehyde. This latter aspect presented something of a problem, for while the G.L.C. method has the advantage of accuracy (Lees & Jago, 1969), its practical application is likely to be limited. On the other hand, colorimetric methods can cause problems over reliability, and hence a method of accurately detecting levels of acetaldehyde in natural yogurt for use on a routine basis was developed by the Faculty of Agriculture and Food Analytical Service. The desire for relative ease of operation implied the use of the minimum of sophisticated equipment, and reliance on readily available reagents. Thus, the technique of Roach and Creavan (1968) was used to prepare the samples for analysis, while a colorimetric estimation modified from Koneca and Dokladalova (1970) provided the method for determining the levels of acetaldehyde.

Material and method

After some preliminary trials, three starters were chosen on the basis of perceived differences in flavour imparted to natural yogurt. These starters were all available in freeze-dried form, and were coded: "CH-1" and "Boll 3" (Chris Hansen's Laboratory, Denmark) and "RR" from NIZO, Holland (courtesy of Dr The. Galesloot). The working cultures were maintained in reconstituted skim milk (9 per cent total solids) and, after an initial incubation at 42°C to around 0·85 per cent lactic acid, were held at +5°C before use. The ratio of *Streptococcus : Lactobacillus* was kept in the region of 3:1 (cell:cell basis), and monitored using a modified Breed smear method (Robinson & Tamime, 1976).

In order to standardise the experimental yogurts, full cream spray-dried milk powder was used, reconstituted to give 16 per cent total solids in the final mix. The milk was then heat-treated at 85°C for 30 minutes and, after cooling to 42°C, was inoculated with an appropriate starter at a rate of 2 per cent v/v. The mix was then dispensed into standard plastic cups (5 oz) and, after capping, the cups were incubated at 42°C for 3½ hours. At this stage coagulation was well advanced, and the cartons of yogurt were transferred to a refrigerator at +5°C. After 24 hours, tests indicated a totally cohesive coagulum, and an acidity of around 1·0 per cent lactic acid; the desired level for consumer acceptability (Crawford, 1962; Davis, 1973). If these initial tests were satisfactory, the remaining cartons were employed immediately for organoleptic appraisal or chemical analysis.

The taste panel for these experiments consisted of a group of 25 students with Middle Eastern or Mediterranean backgrounds. This group was selected on the basis of their known familiarity with natural, unsweetened yogurt, so that flavour preference would to some degree reflect intensity as judged against an "in-built" mental standard; in warmer climates, yogurt tends to have a much sharper and more aromatic flavour. The method of assessment of the three types of yogurt was that of Pearce & Heap (1974), and every taster received the samples in a prescribed order (Fisher & Yates, 1974) and under conditions designed to eradicate bias.

A parallel series of yogurt from the same batch was analysed for acetaldehyde content, and the procedure was as follows. A sample (50 g) of yogurt was weighed into a 500 ml beaker, and mixed with around 100 ml of distilled water.

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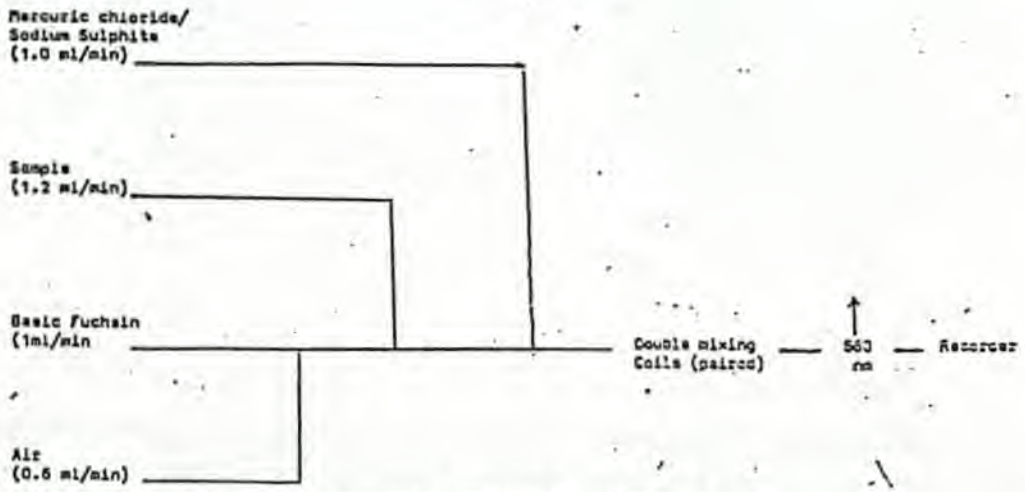

Fig. 1. Diagrammatic representation of the automated procedure for measuring the level of "acetaldehyde" in yogurt

The mixture was then transferred, with careful rinsing, to a 250 ml volumetric flask, and thoroughly shaken. Twenty ml of 5 per cent zinc sulphate solution was then added, followed by 20 ml of 0·3M barium hydroxide. These latter solutions had the effect of clarifying the sample so that, after further agitation and adjustment of the volume to 250 ml, suitable aliquots could be centrifuged (around 3000 rpm for five minutes) to obtain a clear extract.

The colour reagents consisted of:

(a) 0·04 per cent magenta (basic fuchsin—B.D.H./No. 26120)* in 2 per cent sulphuric acid;

(b) anhydrous sodium sulphite (0·354 g) dissolved in an aliquot of a solution of 2·72 per cent mercuric chloride and 1·17 per cent sodium chloride; once the sodium sulphite had dissolved, the aliquot was made up to 100 ml with more of the mercuric chloride/sodium chloride solution.

The "acetaldehyde" level in the clear extract was determined by adding 1 ml of the magenta solution to a suitable aliquot and shaking. One ml of the mercuric chloride/sodium sulphite solution was then added, and the mixture allowed to stand, in the dark, for 25 minutes. The colour intensity was then measured at 560 nm against known acetaldehyde standards. However, once the basic procedure had been tested, an automated system was devised on the lines shown in Figure 1. This system was found to be capable of handling 40 samples an hour, and was especially suitable for acetaldehyde levels of 0·25 μg/ml in the final solution (see table).

*The use of p-rosaniline is no longer acceptable, as the compound has been rated as a "suspected carcinogen"

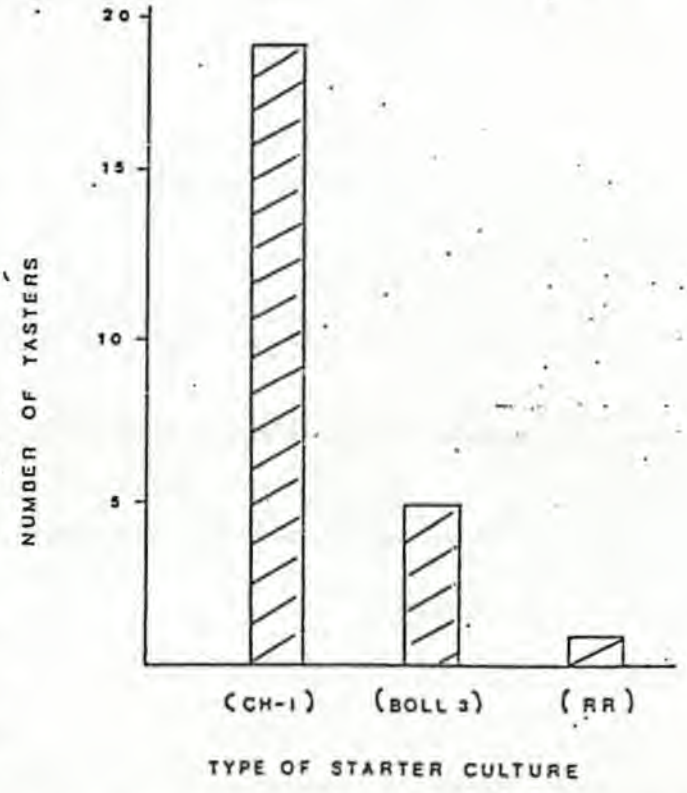

Fig. 2. Overall preferences for particular types of yogurt as judged on appearance, consistency and flavour

Results and discussion

The tasters were asked to score each yogurt as a separate entity, and the means of the overall ratings were employed to indicate sample preference; the weighting employed in the scheme means that overall preference can be broadly equated with flavour preference. The results of the appraisal are shown in Figure 2, and it is clear that the yogurt (CH-1) is the preferred sample, with yogurts (Boll-3) and (RR) faring rather poorly. This scoring implies, of course, no criticism of the less flavoured starters which are highly rated in respect of imparting consistency, but the traditional aromatic flavour of natural set yogurt was clearly best attained, in the view of the tasters, with starter (CH-1).

The results of the acetaldehyde analysis are shown in the table and the correlation with the organoleptic appraisal shown in Figure 2 is quite distinct.

Detected levels of "acetaldehyde" in yogurts produced with different starter cultures; means of ten samples

Starter culture:	Code CH-1	Boll-3	RR
Acetaldehyde (ppm)	37·5 ± 2·3	27·6 ± 1·3	10·4 ± 0·3

Obviously this approach to the estimation of acetaldehyde, like other (Schiff type) colorimetric methods, can be criticised in being non-specific for acetaldehyde. However, it is relevant in the present context that it does provide a rapid, simple determination of those constituents, mainly aldehydic or ketonic in property, that appear to be major contributors to the flavour of yogurt.

If this view is accepted, then the implications of these results are really two-fold, in that:

(a) with relatively experienced tasters, a clear relationship can be established between the level of "acetaldehyde" in natural yogurt and the intensity (and acceptability) of perceived flavour;

(b) the reliability of the method devised to measure "acetaldehyde" levels suggests that it could be employed as a routine method for quality control.

The reason that this latter approach could prove valuable, is that natural set yogurt is probably consumed by a more discerning section of the market as compared with the fruit/flavoured varieties. The implication of this trend is that a discrete yogurt flavour becomes of paramount importance, and a routine method for assessing that a given yogurt starter is behaving correctly in this context could prove attractive.

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Summary

It is a widely held view that carbonyl compounds play a major rôle in producing the characteristic aroma and flavour of yogurt. A key component in this system is acetaldehyde, and a new method of determining the level of acetaldehyde in yogurt has been devised. Like some other colorimetric systems, it is not specific for acetaldehyde, but the simplicity and reproducibility of the method suggests that it could play a rôle in routine quality control. This proposal is supported by a close correlation that was observed between acetaldehyde levels in natural set yogurt, and "consumer" preference as recorded by a taste panel.

Household food consumption October-December 1976

Averaged over the fourth quarter, consumption of liquid milk was almost the same as in the corresponding period of the previous year, at 4·67 pints/person/week. The price increase of 1p a pint in September seems to have resulted temporarily in a slightly reduced level of consumption, but there were signs of recovery towards the end of the fourth quarter. Recorded average weekly purchases of condensed milk were unusually low at 0·12 pints (liquid equivalent). Average consumption of cheese, while higher than in the previous year, declined slightly from 3·79 oz in the previous quarter to 3·71 oz: most of the decline occurring towards the end of the quarter when the average price rose in real terms.

Although consumption of butter, margarine, cooking fats and cooking oils all increased compared with the previous quarter, in total the average of 11·4 oz person/week was nearly 3 per cent lower than in the fourth quarter of 1975. The displacement of butter by margarine throughout 1976 resulted in household purchases of soft margarine moving ahead of those of other margarines, the averages for each type in the fourth quarter of 1976 being respectively 1·80 oz and 1·48 oz compared with 1·21 oz and 1·69 oz a year previously.

The continued weakening in demand for eggs, together with an increase of 7 per cent in their average price, led to a fall in consumption from 4·03 eggs per person per week in July-September, to 3·88 in October-December. — *National Food Survey*, Ministry of Agriculture.

The National Food Survey, conducted on behalf of the Ministry of Agriculture is a continuous sampling enquiry into the domestic food consumption, expenditure and nutrition of private households in Great Britain. By obtaining information simultaneously about the quantities, cost and varieties of food purchased by a random sample of housewives, the Survey enables comparisons to be made of dietary and food expenditure patterns and of average prices actually paid for food, not only in Great Britain as a whole but by different kinds of household. Detailed reports are published annually. The latest volume, published on 2 July 1976, gave information on household food consumption and expenditure in 1974.

"Stilton" cheese copyright

The annual meeting of the Stilton Cheese Makers' Association held in March re-elected for a further term of office Mr R. Watson, chairman, Mr J. Stockdale, vice-chairman, Mr J. Crosher, treasurer and Mr G. Allaway, secretary.

The Association states that over the past six months a number of cases have come to its notice of the unauthorised use of the registered trade name "Stilton". The copyright is vested in the Stilton Cheese Makers' Association. The name "Stilton" can, in law, only be applied to cheese made in the approved and officially recognised manner in the three counties of Leicestershire, Nottinghamshire and Derbyshire and nowhere else in the world. A consistently high standard of manufacture is strictly maintained by this means.

Cultures for yogurt —their selection and use

R K Robinson, Department of Food Science, University of Reading, reports on the importance of selecting the correct culture and incubation temperatures to produce the required yogurt product.

Although the addition of fruit and/or other ingredients tends to mask some of the faults in retail yogurts, there is no doubt that consumers are well aware of differences in overall quality. Variations in texture or consistency are one obvious source of potential dissatisfaction, but the absence of a background flavour of yogurt or an inappropriate level of acidity can also provide causes for complaint.

The precise reason for the appearance of these faults will obviously vary, but in many cases, the problem can be traced back to a lack of attention to the starter culture.

On some occasions, this may simply involve the employment of the wrong type of starter for a particular product/process, but on others, it may be that the essential requirements of the constituent bacteria have been overlooked, or just blatantly ignored.

Thus the culture used is absolutely central for success — not only because a poor starter will invariably give rise to a poor product — but because, conversely, the correct culture, handled in a manner that exploits its potential to the full, can ensure that the end-product has just the right features for its chosen market.

In some respects, this statement is an elaboration of the obvious, but even so, all too often the desire for some alleged improvement in a processing schedule can lead to a total dereliction of fundamental principles. Such neglect is, of course, highly undesirable, because under such conditions, the metabolism desired of the bacteria is unlikely to be manifest, and as a consequence, the consumer will be supplied with an indifferent product.

If carried to extremes, then it is the producer who will suffer not only through a loss of custom but perhaps through total failures in the vat due to phage or other factors.

Concern over the selection and use of starter cultures is, therefore, in the interest of producers and consumers alike.

The aim of this present study was to:

(i) emphasise the very real differences that exist between the various starter-cultures on the market;

(ii) examine the extent to which these differences can be exploited;

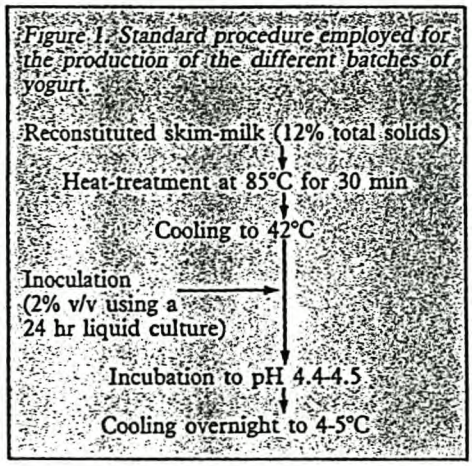
(iii) demonstrate the impact of cultural conditions on the behaviour of some typical starters.

Selection of starter cultures

It is widely accepted that cultures for yogurt can usually be allocated to one of two groups, ie those suitable for set yogurt and those for stirred.

In practice, these categories are not always observed too strictly, but the division does indicate that while some yogurts will have a delicate gel structure after incubation, others will have a more coarse structure that is better suited for the incorporation of fruit. This difference is a reflection of the fact that the bacteria in the latter cultures secrete demonstrable levels of extracellular polysaccharides, while in the former case, the structure of the coagulum is derived almost entirely from the gel formed by the milk proteins.

Figure 1. Standard procedure employed for the production of the different batches of yogurt.



It is important, of course, that this contrast is most relevant at the extremes, and many cultures with intermediate properties, in terms of their ability to impart structure, can be discerned.

The precise type of structure similarly varies with the strains of bacteria present, so that while some stirred yogurts have a smooth, semi-fluid consistency, others have a markedly gelatinous structure.

What these differences mean in terms of levels of, or chemical natures of, the relevant metabolites is not clear, but the implications are that the manufacturer may well be able to select a culture that will provide just the characteristics that are being sought for the product. Equally, it may be possible to match the culture to the process, for the degree of 'set-back', for example, that can be achieved by a coagulum after stirring and cooling can be influenced considerably by the starter employed.

This relationship between the type of starter and the nature of the end-product is, to a degree, self-evident but even so, it is probable that many selections are still made on an almost arbitrary basis. Yet in reality the activity of the starter can influence the entire spectrum of characteristics of the finished yogurt.

In order to emphasise this point, a study was made of the organoleptic properties of three yogurts made with three different starters. Obviously, the cultures were selected in the anticipation that the discernible features of the resultant yogurts would be markedly distinctive, but this deliberate bias does not detract from the validity of the overall concept.

Three types of natural yogurt were produced employing the standard procedure shown in figure 1, and after holding overnight at 4°C, individual cartons of each yogurt were given to a panel of ten assessors; a selection of commercial products were also made available.

The aim of this initial examination was to construct a vocabulary of attributes which could be used to compare the test samples, and the outcome of the debate was as follows:—

Appearance (of broken coagulum)	:	Showing syneresis Smooth Irregular
Aroma	:	Intense Cheese-like
Taste	:	Acidic Creamy Sweet Intense
Mouthfeel	:	Smooth Viscous Slimy Dry
After-taste	:	Sour Dry

This list of attributes was incorporated into a profiling sheet and each assessor was then asked, on an individual basis, to analyse each of the three types of yogurt. An assessor scores the attribute by marking a vertical line on the scale in relation to what they perceive as being the magnitude of the attribute, and the distance of these marks from 'none' is recorded in millimetres. Each assessor was required to examine the three types of yogurt for all sixteen attributes at a single session, and one week later the assessment was repeated with freshly prepared samples.

Analysis of the results indicated that ten of the attributes were appropriate for discriminating between samples, and the relevant data were recorded on the 'star diagram' shown in figure 3. It must be emphasised, of course, that the panel was too small for the conclusions to be totally reliable, but the comparison between the diagrams is, nonetheless, instructive.

Thus, the culture designated as being for set natural yogurt CH_1 gave a strong profile in respect of flavour/acidity and coagulum irregularity (to be expected as the protein gel breaks-up on stirring) while RR produced a smooth, somewhat slimy yogurt with a mild flavour. The profile for B_3 revealed its suita-

bility for stirred yogurt, in that it showed the ability both to produce a discernible yogurt flavour and a smooth, viscous consistency. It was also evident that B_3 gave rise to a more gelatinous coagulum (irregular appearance) than RR, and this difference may indicate that the cultures are secreting different forms of polysaccharide.

The overall conclusion that may be drawn from this exercise is that such profiles can be extremely informative about the properties that a culture can impart to an end-product. It is probable, of course, that the technique and/or vocabulary could be refined further, but even in its present form, the application of this approach could provide information that would:

(a) simplify the process of selecting a culture for a given product;
(b) assist culture manufacturers in the selection of new strains capable of producing more precisely defined characteristics in a yogurt;
(c) allow the enhancement, perhaps, of one specific feature of a yogurt through the use of a standard culture together with a special strain of *Streptococcus thermophilus* or *Lactobacillus debrueckii* sub-sp *bulgaricus (Lac. bulgaricus)*, or indeed a different species entirely.

However, it must be emphasised that even if the process of culture selection could be refined in this way, the anticipated advantages would not be realised unless manufacturers come to accept the dramatic impact that process conditions can have on the performance of any given culture. Obviously, many producers do take this matter very seriously, and as will be shown subsequently, their attention to detail is not misplaced.

Factors influencing culture behaviour

In commercial practice, the design of the plant will to some degree dictate the operating conditions, and clearly these predetermined constraints may well effect the quality of the end-product.

Many other factors can be controlled by the manufacturer, and one such factor is the temperature of incubation. However, this basic parameter, which is absolutely central to the growth and metabolism of the starter culture, often receives scant attention.

In some cases, mechanical difficulties make accurate temperature control something of a problem, but in other instances, manufacturers appear to assume that any temperature between 40 and 47°C will prove acceptable. In reality, this latter approach inevitably leads to all manner of problems and the object of the following investigation was to indicate just why 42°C is usually recommended as the optimum.

Rate of acid development

The procedure employed for making the yogurt was outlined in figure 1, except that on this occasion, the cultures employed were THY 27 and THY 98 supplied by Miles Laboratories Ltd, Slough (UK). The former is one of the group normally recommended for set yogurt, while the latter gives rise to the more viscous coagulum associated with stirred, fruit yogurt.

The temperatures selected for this examination ranged from 30-50°C, and at each temperature, duplicate samples (100 ml) of inoculated milk were incubated for 4 hrs. At the end of this time, the contents of each flask were mixed thoroughly, and two aliquots (10 ml) were withdrawn from each flask for determination of titratable acidity (1). The results are shown in table I, together with those observed with pure cultures of *Str. thermophilus* and *Lac. bulgaricus* from THY 27.

Table I. Mean titratable acidities of test yogurts produced by the cultures indicated, and incubated for 4 hr at the temperatures specified.

Culture	Temperature of incubation (°C)					
	30	35	40	42	45	50
THY 27	0.25	0.3	0.62	0.75	0.80	0.35
THY 98	0.29	0.45	0.73	0.75	0.78	0.45
Str. thermophilus	0.21	0.28	0.32	0.35	0.30	0.25
Lac. bulgaricus	0.25	0.29	0.35	0.35	0.40	0.35

Table II. Mean depths of penetration (mm) of a standard spindle into set yogurts incubated at different temperatures, and with the cultures indicated; the time interval was, in every case, 5 seconds.

Temperature (°C)	Culture	
	THY 27	THY 98
35	320	360
40	360	375
42	345	400
45	250*	480
50	160*	220*

* abnormal coagulum structures

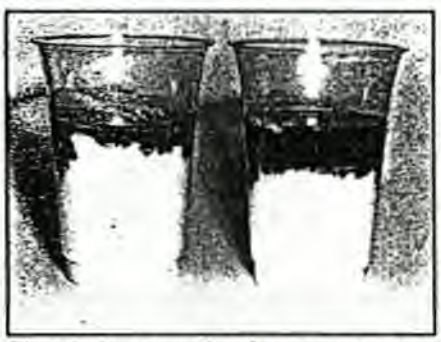


Figure 2. Some examples of set yogurts produced at elevated temperatures of incubation: (left) 45°C; (right) 50°C. Note: the whey is stained purely to provide contrast.

The precise levels of acidity do, of course, merely reflect the experimental conditions applied, but it is of note that culture THY 27 did show a fairly definite optimum at 45°C. The reaction of THY 98 was however, much less pronounced and as expected, the single species behaved independently; the superior activity of *Str. thermophilus* at 42°C was clearly evident. Nevertheless, the superficial conclusion from these results could be that a temperature higher than the conventional 42°C would be advantageous.

There is, however, more to yogurt production than the mere generation of acidity, and the attainment of an acceptable consistency is an essential target for any responsible manufacturer. The incorporation of stabilisers can, of course, remove some of the pressure for success in this area, but even so, the essential characteristics of a yogurt have to be provided by the milk proteins, and hence the effect of incubation temperature on the coagulation of these components is a consideration of some importance.

Nature of the coagulum

The initial examination was carried out on set yogurts using a standard 'penetrometer' (2), and the influence of incubation temperature on the relative strengths of the resultant gels is shown in table II.

In each case, the incubation times were adjusted to give a standard pH of 4.4-4.5 at the onset of cooling, and all readings were carried out after the samples had been subject to overnight refrigeration.

In a test of this type, the penetrometer is measuring the resistance of the protein network to a weight under gravity, and hence the data provide information as to the degree of bonding within the network.

What is evident, therefore, is that the two cultures are influencing their respective gels in different ways. Thus, the coagulum produced by THY 98 decreases in strength as the temperature of incubation is raised, while in the presence of THY 27, the nature of the gel does not alter significantly until the upper range of temperatures. This latter point is borne out by the typical samples shown in figure 2, which confirm incidently that, irrespective of any other benefits, temperatures of 45°C or over cannot be employed to manufacture an acceptable set yogurt using a standard culture.

The same conclusion appears equally relevant for 'viscous' cultures, although in the latter case, the reasons are somewhat different. Thus, the pattern observed in the presence of THY 27 suggests that the firmness of the coagulum is dependent on protein-protein interactions, whereas in the viscous yogurt (THY 98), the extra-cellular polysaccharides would appear to be a major influence. This impact can be deduced from the fact that as microbial activity increases with temperature, and hence, presumably, polysaccharide production as well, so coagulum strength declines. The implications of this decrease are, in the present context, that:

(i) that the carbohydrate material was reducing the level of protein interactions, a view supported by the work of Tamime *et al* (3) and
(ii) that the texture of the yogurt derived from culture (THY 98) was heavily dependent upon the properties of the polysaccharide.

The possible validity of this latter point was investigated by examining the effect of incubation temperature on the viscosity of the same yogurts.

Consistency of stirred yogurts

The basic procedure for making the samples was followed, the only deviation being that the inoculated milks were incubated as 500 ml batches in covered beakers. At the

desired pH, the individual batches were cooled overnight to 4-5°C.

Next morning, each sample was stirred manually to a smooth, even consistency, and the viscosities of the cool yogurts were measured with a Brookfield Viscometer and helical-path attachment. The results are shown in table III.

What is noticeable about these data is that the viscosities recorded for the coagula produced by THY 27 were, except for the abnormal gel produced at 50°C, higher than those recorded in the presence of THY 98.

The most likely explanation of this phenomenon revolves around the effect of shearing on the disparate coagula, and the basic hypothesis is shown diagrammatically in figure 4. The behaviour of the less viscous coagulum (THY 98) is represented by (a), and it is envisaged that the final yogurt

Table III. Mean viscosities (in centipoises × 10,000) of stirred yogurts incubated at different temperatures and with the cultures indicated.

Temperature (°C)	Culture THY 27	THY 98
35	279	113
40	292	133
42	339	146
45	306	206
50	239	253

consists of 'large' fragments of protein gel surrounded by a matrix of water/polysaccharide. However, the same shear force might well cause the gel of THY 27 to disintegrate into a virtually homogeneous system (c); an intermediate situation is shown in (b).

If this view is acceptable, then the lower viscosity recorded for the yogurt based on THY 98 can be explained by the fact that the rotating spindle of the viscometer is, in reality, measuring primarily the resistance of the water/polysaccharide mixture.

In the other yogurt (THY 27), the resistance is supplied in equal measure by the mall' gel pieces and the surrounding fluid, and as a result, the apparent viscosity of the product is higher. Unfortunately, systems of this latter type are highly shear dependent, and the enhanced viscosity recorded in this test would not be sustained during any normal manufacturing procedure.

Although the protein gel of the stirred yogurt makes an essential contribution to the organoleptic character of the product, it would appear that the observed viscosity is supplied mainly by the secreted polysaccharide. These same materials offer, in addition, some protection to the gel fragments against further breakdown, so that the coagulum becomes resistant to the stresses of mechanical agitation.

The role of certain stabilisers would also be accommodated by the same scenario, for if the concentration of polysaccharide in the aqueous phase is raised, so the viscosity of the yogurt might be expected to increase as well.

This conclusion does, of course, open a further avenue for the exploitation of starter cultures, for a system that could enhance viscosity without introducing a 'slimy' texture could prove attractive to consumer and producers alike.

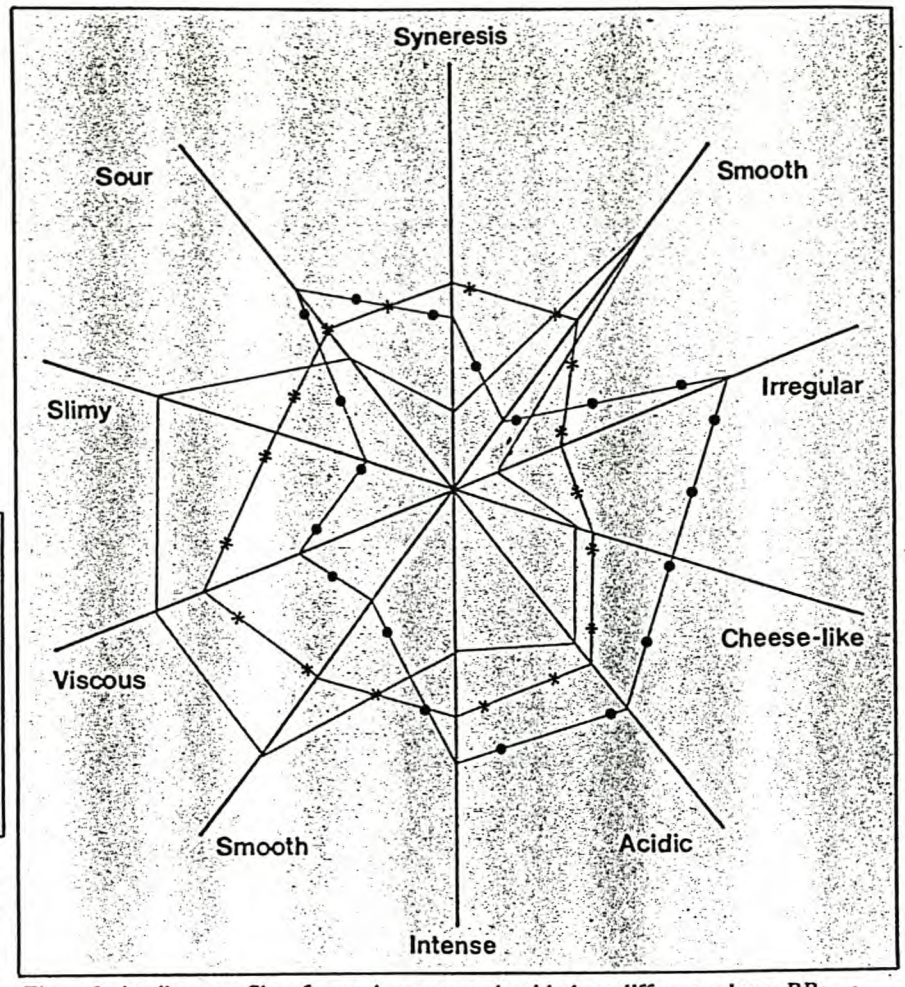


Figure 3. Attribute profiles of natural yogurts made with three different cultures RR:—; B3:; CH1:●.*

Footnote: Culture RR was made available through the courtesy of NIZO, Holland and cultures of CH1 and B3 were provided by Chr. Hansen's Laboratory, Reading, Berks.

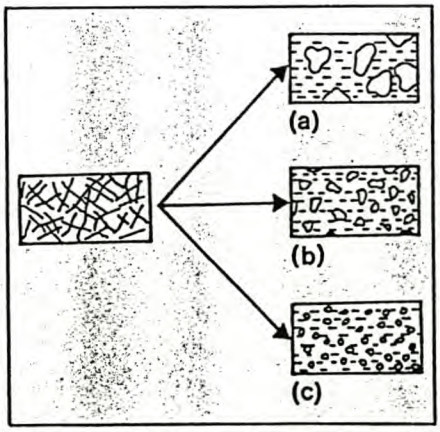


Figure 4. A diagrammatic representation of the possible effects of shear on the coagula of set yogurts made with different types of starter culture.

The feasibility of the idea was examined by producing yogurt employing the standard procedure, except that the inoculum included an additional strain of either *Lac. bulgaricus* (LBF3) — known to secrete copious amounts of extra-cellular polysaccharide, or a similarly active strain of *Str. thermophilus*. The viscosities of the test yogurts were recorded in the normal manner, and the results are shown in table IV.

Table IV. Mean viscosities (in centipoises × 10,000) of stirred yogurts inoculated with the cultures indicated; temperature of incubation — 42°C.

	Culture/Radio of addition A	A/B (3:1)	A/B (1:1)	A/C (3:1)	A/C (1:1)
Viscosity	146	186	345	259	234

Code of cultures: A — Mixed culture — THY 98
B — *Lac. bulgaricus* — LBF3
C — *Str. thermophilus* — SCF1

The behaviour of the yogurts incorporaing LBF3 would appear to confirm the view that selectively enhancing the level of secreted polysaccharide would have a beneficial effect on viscosity, and that the nature of the stirred coagulum (THY 98) may well be as portrayed in figure 4.

It is equally evident that the nature of the polysaccharide is critical, for although the material secreted by SCF 1 may well alter the organoleptic character of the end-product, its impact on viscosity *per se* is somewhat marginal. Obviously, any conclusions concerning the nature of the polysaccharide must be speculative, but the observed differences between the final products does serve to emphasise further that

culture selection deserves special attention.

This brief examination of the influence of temperature on the physical properties of yogurt also provides a clear indication as to why 42°C should be regarded as the optimum for production. Thus, in the case of the set yogurt, the damage caused by a too rapid rate of acidification is clearly visible, but even for stirred yogurt, it could be argued that 42°C represents the point at which the coagulum demonstrates an optimum blend of elasticity and viscosity.

The impact of these two components on consumer appeal cannot, in the absence of a detailed appraisal, be judged with any degree of accuracy, but certainly a decision to employ a temperature higher than 42/43°C must be viewed with suspicion.

Further considerations

It is clear from the above discussion that, although a temperature of 42°C or below is essential for the manufacture of set yogurt, an increase to 45-46°C might just be tolerable for producing a stirred yogurt.

However, the emphasis must be on the word 'might', and in practice, it is probably that 42°C remains the ideal compromise for all yogurt production by the 'short-set' method. This conclusion can be justified by the facts that:

(i) the optimum base consistency for a stirred yogurt may well be achieved when the imparted structure represents a balance between the natural bonding of the milk proteins and the viscosity of the polysaccharides secreted by the culture. A tentative analysis of the above data suggests that this balance is most likely to be achieved with an incubation temperature of 42°C; and

(ii) the employment of a temperature of 45°C or over clearly places considerable stress on the streptococcal component of any normal culture, and the implications of this situation could be serious.

Thus, during normal production, the stress imposed on *Str. thermophilus* by an elevated temperature may, for example, result in:

(i) some loss of typical, yogurt flavour in the end-product (4); and

(ii) a reduction in the number of viable cells of *Str. thermophilus* in the retail yogurt,

but neither of these faults need, of course, be causes for immediate concern.

However, any stress applied to a population of bacteria tends to render the individuals more susceptible to other inhibitory factors, so that growing a yogurt culture at the inimical temperature of 45°C may well increase the risk of failure. Inhibitory substances in the milk, for example, might be expected to have a more dramatic impact at 45°C than at 42°C, but of even greater significance could be the possible relationship between sensitivity to phage and temperature of exposure.

It must be admitted, of course, that reported incidents of bacteriophage activity against *Str. thermophilus* have, as far as the UK is concerned, been minimal, but the precise reasons for this fortunate situation are far from clear.

The combination of high standards of hygiene and the severe heat-treatment of the yogurt milk is usually seen as the most plausible explanation, and certainly a high-temperature-holding process, eg 85°C for 30 minutes, offers adequate assurance that phage particles will not be carried-over into the vat.

This confidence must be tempered, however, in the light of findings that many isolated bacteriophages of *Str. thermophilus* have a narrow host range (5) so that, in reality, many incidents of phage attack may remain undiagnosed, ie the manufacturer simply avoids the impending problem by changing to a different culture.

This solution is, of course, perfectly reasonable, so long as:

(i) the standard of hygiene in the plant in question is high enough to prevent the rampant proliferation of alternative phage types; and

(ii) the susceptibility of the introduced strain of *Str. thermophilus* to phages does not increase.

It is this latter point that could be relevant in the present context, because certainly with mesophilic cultures, there is evidence that the application of heat-stress to a host will render it susceptible to types of phage to which it was previously resistant (6).

Similarly, the rate of proliferation of certain phages can increase under temperature conditions that disadvantage the host, and it is possible that this situation is analogous to the incubation of *Str. thermophilus* at 45°C.

If this surmise is valid, then a further reason for the absence, to date, of major phage problems in the yogurt industry could be the willingness of manufacturers to adhere to the conventional practice of fermentation at 42°C. Obviously it is not possible to state categorically that the employment of 45°C for yogurt-making increases the likelihood of infection by phage, but even so, the possibility cannot be discounted.

Conclusion

Although the number of cultures examined during this study has been limited, the work has served to emphasise the advantages of selecting the correct culture for the product in question.

It has confirmed also that incubation temperature can have a considerable influence on the activity of a typical, mixed yogurt starter, and furthermore, that 42°C does represent the optimum, and indeed acceptable maximum, temperature for the synergistic growth and metabolism of the constituent species.

Acknowledgements

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A Marschall International Dairy Symposium Paper

Cultures & quality

Mouthfeel is increasingly used by consumers as a criterion for evaluating stirred yogurts. The University of Reading's Richard Robinson looks at the role played here by cultures and coagulants

There are many varieties of yogurts and bio yogurts, and individual decisions to purchase will be governed by many factors. Range of flavours, convenience and price are often quoted as the decisive factors. But, as access to supermarkets improves, sensory quality increasingly determines final selection.

In practice, distinguishing brands of stirred yogurt on the basis of fruit flavour is not easy for the non specialist, and quality is more likely to be recognised in terms of attributes like viscosity and perceived mouthfeel. Consumers will always differ in terms of their expectations, but we might assume that the majority prefers a spoonable product with a smooth, creamy feel on the palate. The role of cultures and coagulants here is crucial.

Selection of cultures

For set yogurts, non polysaccharide-producing cultures provide the best option, because the presence of polymers tends to disrupt the establishment of protein-protein bonds and gives rise to a weaker gel. However, the situation is different with stirred yogurts – where imparted viscosity is probably more important than coagulum strength.

The ability of selected strains of *Streptococcus thermophilus* (1, 2, 3) and *Lactobacillus delbrueckii* subsp *bulgaricus* (4, 5) to secrete extra-cellular polysaccharides has long been exploited by yogurt manufacturers seeking more attractive stirred products (6, 7, 8). Similarly, the fact that some strains produce capsular polysaccharides (8, 9) and that others release ropy materials like glucans (1) has received some attention.

In practical terms, this contrast means that a culture may: (a) impart a slightly gummy texture to the product; (b) give a gel that is fairly resistant to applied stress; and (c) encourage a degree of viscosity recovery in the carton. Alternatively it might: (a) give a yogurt with a somewhat slimy mouthfeel; and (b) produce a coagulum with lower structural stability in terms of applied stress.

These differences appear to reflect the contrasted natures of the polysaccharides in that, while both types will increase the viscosity of the aqueous phase of a stirred yogurt, the capsular types are not damaged by the stresses

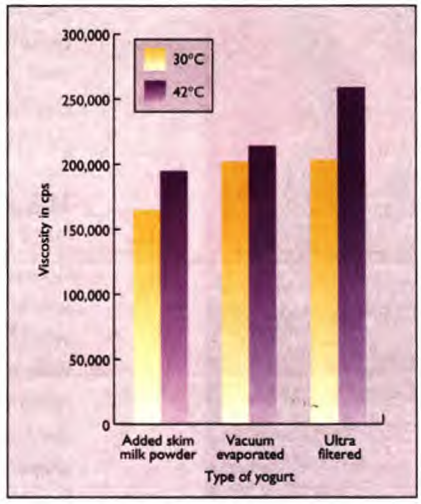

Figure 1. Effect of incubation temperature on the viscosity of stirred, natural yogurts (16% total solids)

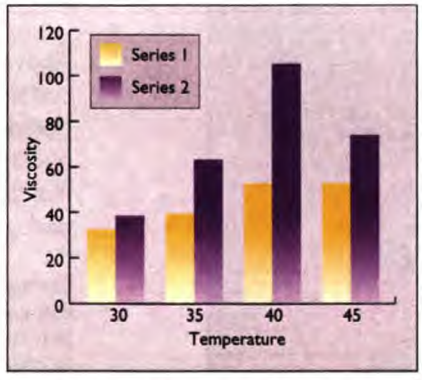

Figure 2. Effect of incubation temperature on the viscosity of stirred, natural yogurts (16% total solids), with (maroon) and without (blue) exposure of the yogurt to a holding period at 25°C for three hours at the end of incubation

of stirring or pumping. Consequently, any viscosity imparted to the product is retained during processing – and may even be enhanced on subsequent standing. However, the so-called ropy polysaccharides are prone to structural damage during processing and, as the chemical bonds do not reform, the loss of viscosity is permanent (9).

The potential for manipulating the properties of yogurt which arise from these disparate patterns of behaviour has yet to be fully exploited (10). Indeed, it may not be possible to refine the use of polysaccharide-producing strains until the structures of the polysaccharides themselves are more clearly defined.

For example, there is general agreement that glucose and galactose are the important components of the polysaccharides secreted by *S thermophilus* and *L delbrueckii* subsp *bulgaricus*. But, as is evident from Table 1, the relevance of other sugars is far from clear. The extent of branching is also subject to debate (1), yet this topic of structure could be extremely important if extra-cellular polysaccharides are to be exploited to generate the product consistency consumers desire.

Handling of the coagulum

Before considering the polysaccharide option in more detail, it must be emphasised that yogurt makers have a pivotal role in maximising the potential of the cultures on offer. For example, success with a chosen culture depends: (a) on inoculation of the milk so that the balance between the constituent species is correct; (b) on the selection of the correct incubation temperature; and (c) on finding the best way to handle the coagulum.

However, there appear to be few definitive studies of the conditions necessary to achieve a specified set of characteristics in a retail yogurt.

Temperature of incubation is critical. An experiment, the results of which are shown in Figure 1, suggested that more rapid acidification of the milk at the higher temperature has a beneficial impact on gel strength/viscosity, probably because more protein-protein bonds are formed, which leads to a firmer gel.

It is also relevant that the culture of *L delbrueckii* subsp *bulgaricus* employed in the tests was one that synthesised a capsular polysaccharide, and hence viscosity related to the number of cells present and not to the volume of polysaccharide secreted (6).

The conclusions of the experiment are highlighted in Figure 2, which shows the viscosities of yogurts made with a ropy culture and with incubation at the temperatures indicated. In total, two series of trials were completed. In Series 1 (blue blocks), the yogurts were incubated to pH 4.3 and then chilled. The yogurts in Series 2 were held at 25°C for three hours between the end of incubation and the onset of chilling.

The evidence would seem to indicate that, for this specific culture,

polysaccharide secretion is not improved by raising the temperature of incubation. But – provided that the culture has been grown at its optimum temperature to generate high cell numbers – a resting period at 25°C or thereabouts encourages the synthesis of polysaccharides and the generation of additional product viscosity (6). This is, perhaps, a culture characteristic which could be exploited further.

The handling of the coagulum after incubation will, of course, affect the consistency of the retail item as well, and it is interesting that the Danish Research Institute (11) has recommended severe agitation of the coagulum immediately after the cessation of incubation. Provided that there are enough total solids, that the selected stabiliser is appropriate and, perhaps, that the culture produces a capsular polysaccharide, then the end-product remains smooth and stable throughout its shelf life.

Unfortunately the procedure destroys the coagulum, so that – unless a technique becomes available for re-establishment of a gel structure – a spoonable product will not be achieved. If a ropy culture is preferred, then more gentle handling of the coagulum at temperatures of 15-20°C is regarded as the more desirable option. Generally it is agreed that, once the protein-protein/carbohydrate-carbohydrate/protein-carbohydrate bonds within the gel are broken, they cannot be reformed.

Clearly, this approach may prove impossible in a large factory with yogurt flowing through a myriad of pipes and pumps. As such, if yogurts of mediocre quality are to be avoided, then alternative strategies must be sought.

Future prospects

If it is assumed that manufacturers are going to continue to economise on levels of casein, that the coagulum will always be damaged during fruit blending and that the cost of the best stabilisers will continue to rise, then the remaining option for improving the quality of stirred fruit yogurts lies with the starter cultures.

For example, it will soon be possible to transfer the gene(s) for polysaccharide production from one starter organism to another (12), or to replicate the genes within a strain to increase the level of material secreted.

But, as mentioned previously, the chemistry of these microbial polysaccharides does not appear to be well established. In the absence of such information, an alternative approach would be the production of cultures capable of generating polysaccharides with already well-defined characteristics. In this way, the behaviour of the material could be predicted.

For example, if the genes for xanthan gum formation in *Xanthomonas campestris* could be transferred into a strain of *S thermophilus*, then a detailed dossier covering the performance of the polysaccharide could be provided along with the culture (13). Similarly, the genetic sequence responsible for the production of gellan gum in *Sphingomonas elodea* might be amenable to transfer and, once again, the behaviour of the polysaccharide would be entirely predictable.

Furthermore, if the properties of the polysaccharide secreted by a given culture were well defined, then the exploitation of carbohydrate-carbohydrate or carbohydrate-protein interactions becomes a distinct possibility.

Thus, if a culture capable of secreting xanthan gum was used to produce the yogurt base and the fruit purée was stabilised with guar gum, then mixing the fruit into the white base would generate a viscosity dependent on polysaccharide-polysaccharide interactions alone.

Synergistic behaviour is observed also between xanthan gum and locust bean gum, and again the interaction produces a beneficial increase in the viscosity of the aqueous phase. If a strain of *S thermophilus* could produce xanthan gum and *L delbrueckii* subsp *bulgaricus* secreted gellan gum, then additional modifications of the end product might be feasible.

More specifically, it might be relevant that both the xanthan gum/locust bean gum and xanthan gum/gellan gum combinations have a tendency to gel (13) so that, while these materials are essentially thickeners of the aqueous phase, the proposed interactions might give rise to yogurt with a spoonable consistency. If this scenario became reality, then consumers would benefit from the availability of more attractive retail products, while manufacturers would gain from the flexibility to handle yogurt gel in ways that currently cause unacceptable damage.

Whether such options would be permissible under current legislation is a matter for conjecture. It appears, at present, that gene transfers are only permitted between strains of starter culture 'Generally Recognised As Safe' (GRAS). For example, it would seem that an allele for maltose fermentation can be transferred – so long as any markers are inert – from a strain of *Lactococcus lactis* to a strain of *Lactococcus cremoris* (14).

Whether or not the authorities will allow this practice to be extended to intergeneric situations remains to be seen and, similarly, the boundaries of GRAS will need to be explored in more depth.

An additional dimension will be provided by the reaction of the public since, as yet, little media attention has been paid to the possibility that genetically modified bacteria could be present in cheese or yogurt. ■

Table 1. Sugars reported present in polysaccharides secreted by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*

Culture	Sugar								
	Gal	Glu	Fru	Rha	Man	Xyl	Ara	GalA	Neu
Strep thermophilus	+	+	-	-	-	-	-	-	-
	+	+	-	+/-	+	+/-	+/-	-	-
	+	+	-	-	-	-	-	+	-
	+	+	-	-	-	+	-	-	+
	+	+	-	+/-	+/-	-	+/-	-	-
Lac delbrueckii subsp bulgaricus	+	+	-	-	+	-	+	-	-
	+	+	-	-	-	-	-	-	-
	-	+	+	-	-	-	-	-	-
	+	+	-	+	-	-	-	-	-

Gal = galactose; Glu = glucose; Fru = fructose; Rha = rhamnose; Man = mannose; Xyl = xylose; Ara = arabinose; GalA = galactosamine; Neu = neuramic acid + = present; - = absent; +/- = trace reported.

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PRODUCT DESIGN

The heathy image of yoghurts continues to make it a popular product with consumers. Dr Richard Robinson explains what is involved when formulating bio-yoghurts and fermented milks.

Yoghurt

Certain aspects of the 'recipe' for making yoghurt or any similar fermented milk cannot be altered, either because there are stipulations in National Food Regulations that cover specific compositional features, or because the organoleptic properties expected by consumers of set or stirred products can only be met using a traditional formulation.

For example, it is always assumed that yoghurt will be manufactured from the milk of one or more available mammals, eg. cows, sheep, goats or buffalos, and most countries lay-down precise compositional standards with respect to levels of butterfat and milk-solids-not-fat (MSNF) that must be present in natural yoghurt. Similarly, the anticipated consistency of a set yoghurt or viscosity of a stirred yoghurt can best be achieved by exploiting the gellation properties of casein when exposed to the action of lactic acid released by selected starter cultures which, incidently, must be detectable as 'abundant and viable' in the retail product. However, despite the apparent constraints of this essential framework, scope for innovation still exists.

Chemical Composition

Fat: The level of fat in yoghurt reflects, to a large extent, the proposed market, and the minimum value needed to provide a desirable 'mouthfeel' in, for example, a stirred fruit yoghurt is 1.2 - 1.5 per cent; 'very low fat' yoghurts, ie. < 0.5 per cent butterfat, can be relatively insipid and uninspiring. The incorporation of fat replacers, like Simplese _ 100 (NutraSweet Europe, France) which is manufactured from whey protein to give a material with a particle size similar to milk fat globules, can compensate for a reduction in fat, but the use of vegetable oils to provide 'mouthfeel' has yet to be exploited in fermented milks, mainly because the flavour of even refined oils is difficult to mask, as is the slightly 'oily' texture. It may be relevant though that concentrated yoghurts - 23 per cent total solids - are invariably served with olive oil, so there could be a market for a 'yoghurt-dip' in which the butterfat is replaced with an oil.

Protein: Raising the protein levels above the normal 3.3 - 3.5 per cent found in cows' milk is essential in anything other than a fermented milk drink, for it is the coagulation of the casein that provides the basic gel upon which the structure of all yoghurt-like products depends.

Financial considerations usually mean that elevation of the protein is achieved by: (i) the evaporation of water from liquid milk under vacuum; or (ii) the removal of water from liquid milk by ultra-filtration, but fortification of base milk with skim-milk powder (36 per cent protein), sodium caseinate (87 per cent) or a high-protein skim-milk powder (55-75 per cent protein) can be equally effective.

Although these latter powders are excellent for improving the gel strength of set yoghurt or the viscosity of stirred yoghurts, they are expensive options. 'Diluting' high-casein powders with demineralised whey powder can reduce costs, but the functional properties imparted to yoghurt by whey proteins are always inferior to those arising from casein.

Carbohydrates and stabilisers

Polysaccharides: Except in natural yoghurts where authenticity is protected by regulations, thickening of a product with a water-absorbing plant gum or polysaccharide - addition rate of 0.5 - 0.75 per cent - is extremely popular and cost effective. Thus, guar gum, locust-bean gum, as well as the carrageenans and cellulose derivatives, are long-chain polysaccharides composed of regular arrangements of monosaccharide units, and it is this structure that gives them the ability to bind water and nullify any loss of viscosity attributable to a low protein content.

The use of such 'stabilisers' is widespread for both 'spoonable' and drinking fermented milks, and starches/modified starches that can mimic, to a large extent, the properties of these gums are also available; a significant price differential is ensuring that starches are extremely competitive alternatives. Apart from providing a satisfying 'mouthfeel' and viscosity to stirred fermented milks, polysaccharides can also act to stabilise the structure, and hence are valuable in preventing the leakage (syneresis) of whey onto the surface of set or stirred products during transport or storage.

Oligosaccharides: The use of polysaccharide molecules smaller than starch - often referred to as oligosaccharides - is still being explored, for a material like inulin has some useful properties. In the first place, it can enhance the viscosity of a stirred product, although to a lesser extent than a polysaccharide but, equally important, it can act as a stimulant to the microflora of the large intestine. Thus, while inulin is not hydrolysed by enzymes secreted by the human digestive tract, it can be metabolised by the Bifidobacterium spp. that inhabit the walls of the colon.

As these same bacteria are essential for the healthy functioning of the colon, their possible stimulation by the ingestion of inulin is regarded as beneficial. Whether other oligosaccharides isolated from plants or derived from fermentation processes will have similar functions remains to be established, but their role as ingredients of fermented milks could become important.

Mono/Disaccharides: As most fermented milks contain milk solids at levels above those in normal milk, the lactose content of the retail product will be 5 - 6%, with the exact figure depending upon the degree of fortification and the extent to which the lactose will have fermented into lactic acid. However, the residual lactose does not alter the sweetness of a product, and hence sucrose at levels of 5 - 6 per cent has to be added to the types of fruit yoghurt favoured in industrialised countries.

Invert sugars with up to 90 per cent of sucrose converted to glucose and fructose offer a sweetening power equivalent to sucrose and, with some high fructose syrups, the imparted sweetness can be much higher than sucrose. Artificial sweeteners like saccharin and cyclamate can be used to provide intense sweetness without the calories associated with sugars, but it should be noted that: (a) with fermented milks, sugars offer the advantage of giving extra body to the product; and (b) despite regulatory approval, some consumers can suffer adverse reactions following the intake of artificial sweeteners.

Technological Considerations

Whatever the precise formulations, blending of the above ingredients will give base milks with 15 - 16 per cent MSNF for the manufacture of a natural set yoghurt, or 12 - 14 per cent MSNF - along with sugar and stabilisers - for the production of a stirred fruit product. These base milks will then have to be homogenised to stabilise the milk fat and incorporate any dry ingredients, and be heat treated at 90 - 95°C with a holding time of between two and seven minutes.

The latter step is essential to modify the structure of the milk proteins so that the gel formed during fermentation is soft in nature rather

than granular, but it serves also to kill any vegetative micro-organisms that might be present and de-aerate the milk; these latter points assist the growth of the starter culture.

The milk is then cooled to 42°C for inoculation with the thermophilic starter cultures used for yoghurt and similar products (see **Table 1**), or 30°C for those mesophilic cultures that include species of Lactococcus. Incubation will take place in retail cartons for set products, or in bulk tanks for milks that are going to be stirred, with or without the addition of fruit, prior to filling into cartons.

After 4 - 5 hours, a typical yoghurt fermentation will be complete, ie. the acidity of the milk will have risen to 1.2 - 1.4 per cent lactic acid (around pH 4.2 - 4.3), and the total bacterial population of starter origin may well exceed 2.0 x 108 cells ml-1. At this acidity, which is probably the level preferred by most consumers, the milk proteins will have coagulated to form a firm gel. The product must be cooled if over-acidification is to be avoided. For buttermilk or kefir, a slightly lower acidity could be expected following overnight incubation at 30°C.

Cultures for fermented milks

The cultures used most widely in the manufacture of fermented milks are shown in **Table 1** and, currently, the most popular routes for their addition are: (a) production of a liquid 'bulk starter' using reconstituted skim-milk powder and a freeze-dried or frozen culture purchased from a supplier; the process milk will receive a two per cent v/v inoculation of pre-fermented bulk starter (pH around 5.0); or (b) the purchase of a concentrated frozen or freeze-dried culture that can be added direct to the process milk. For sheer convenience, the latter route is becoming more popular, and it is essential for the production of 'bio-yoghurts' where the balance between, and total number of, the various species is critical.

The mesophilic group of bacteria, mainly lactococci, give rise to products with a distinctive 'buttery' flavour that arises from the high levels of diacetyl released by some components in the cultures. Kefir and Koumiss also share some of the same flavour notes, but the mixture of micro-organisms that produce these acidic, mildly alcoholic (0.3 - 0.8 per cent alcohol) drinks gives rise to wide variations in flavour.

However, most fermented milks are produced with cultures consisting of bacterial species with temperature optima of 37 - 45°C. Yoghurt is the most popular product made with thermophilic cultures, and one or both of the same two species (see **Table 1**) are often to be found in the so-called 'health-promoting' products as well. In the 'bio-yoghurts', for example, the traditional yoghurt bacteria acidify the milk rapidly and provide the flavour and physical characteristics expected by the consumer of a yoghurt-like product, while the other species are included to provide the alleged 'health benefits'.

This latter concept arises because the distal end of the small intestine

TABLE 1. THE PRINCIPAL CULTURES EMPLOYED IN THE PRODUCTION OF FERMENTED DAIRY PRODUCTS

	Bacteria of Starter Origin	Main metabolite(s)
Traditional Products		
Buttermilk,	Lactococcus lactis sub-sp.lactis	lactic acid
Cultured cream	Lactococcus lactis sub-sp.lactis biovar diacetylactis Lactococcus lactis sub-sp cremoris . Leuconostoc mesenteroides sub-sp. cremoris	diacetyl acetoin extra-cellular proteins (some cultures)
Yoghurt	Streptococcus thermophilus and Lactobacillus delbrueckii sub-sp. bulgaricus	lactic acid aldehydes ketones, extra-cellular polysaccharides
Kefir	Lactococcus spp., Lactobacillus spp.,	lactic acid
Koumiss	Acetobacter spp. and various yeasts, inc. Candida kefir	acetic acid alcohol
More recent Health-promoting Products		
Drinks		
Acidophilus Milk	Lactobacillus acidophilus	lactic acid alcohol
Yakult	Lactobacillus casei sub-sp. shirota	lactic acid
Future products	Lactobacillus rhamnosus Lactobacillus reuteri Lactobacillus casei	bacteriocins are secreted by some species
Yoghurt-like Products		
'Bio-yoghurts'	Lactobacillus acidophilus Bifidobacterium spp. Streptococcus thermophilus and, sometimes, Lactobacillus delbrueckii sub-sp. bulgaricus.	lactic acid acetic acid extra-cellular polysaccharides

Footnote: (i) The species of Bifidobacterium may be selected from Bifidobacterium bidium, Bifidobacterium longum, Bifidobacterium adolescentis and Bifidobacterium infantis, all of which occur naturally in the intestines of humans. The use of Bifidobacterium animalis (non-human origin) is permitted in some countries.(ii) Kefir and koumiss have long been regarded in Eastern Europe as having special therapeutic or prophylactic properties.

and the colon of the adult human contains millions of bacteria g-1 of content or cm-2 of wall surface and, while some of the inhabitants like Lactobacillus acidophilus and ifidobacterium spp. are beneficial. other constituent species produce a range of phenolic compounds, such as sketol and indole, which could damage living tissue.

Indeed, there is concern over their possible involvement in the initiation of cancer in the lower intestine.

Yoghurt is the most popular product made with thermophilic cultures, and one or both of the same two species are often to be found in the so-called 'health-promoting' products as well as in bio-yoghurts

Any process that tends to suppress the production of such compounds could, therefore, be advantageous, and it is widely believed that the regular ingestion of high numbers of Lactobacillus spp. and Bifidobacterium spp. of human origin helps to establish a more desirable microflora in the intestine.

Final processing

For set yoghurt coagulated in the retail cartons, cooling can be achieved by blowing cold air through the incubation room, or by carefully transferring groups of cartons in their retail trays to a chill room at 2 - 4°C. In-tank cooling of the base with chilled water (2°C) or pumping through a plate or tubular cooler is practiced for stirred/drinking fermented products and, as most fluid products are further processed, the initial cooling is usually to 15 - 20°C. In-tank mixing of the fruit or other flavouring mixture (10 - 15 per cent v/v) can be used, or the yoghurt base and the fruit can be fed through a blending device directly into cartons which are then chilled to 2 - 4°C.

The physical form of the fruit is usually frozen or heat-treated, and while the final choice may be based on price and/or perceived quality, it is essential that the materials are free from yeasts and moulds. Thus, fermented milks of the correct pH should be almost free from bacteria of non-starter origin, and hence microbial spoilage within the anticipated shelf-life of the product is likely to involve fungi. Consumer complaints about excessive acidity are usually indicative of post-production acidification by starter bacteria following temperature abuse of the product somewhere in the chill chain.

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YOGHURT MANUFACTURE

some considerations of quality

THE production of yoghurt is essentially a biological process, and hence subject to the degree of variability associated with any 'living system'. Thus, the quality of the end-product can be influenced by such factors as the chemical composition of the raw milk, the nature of any additives, as well as the conditions selected for manufacture, while lastly, of course, the entire process is dependent on the correct activity of living bacteria. It is hardly surprising, therefore, that the process can, at times, prove temperamental, particularly as ultimate control of the manufacturing schedule rests in the hands of yet another biological component, man.

Nevertheless, while the more obvious deficiencies are both predictable and easily rectified with proper process control, some of the more irritating variations in product quality appear to arise with alarming spontaneity. One such fault is the occurrence in stirred yoghurt of non-dispersable particles referred to as 'nodulation' or 'lumpiness' [1] or 'graininess' [2]. The exact cause of this fault has long been the subject of speculation, and it is the aim of this paper to consider whether current knowledge of the nature and origins of nodulation can be employed to help a manufacturer to avoid this most tedious of problems.

The nature of the problem

Although the presence of nodules does not affect the nutritional value or organoleptic quality of yoghurt, the 'white specks' showing up against the coloured background of a fruit/flavoured product can give the retail material a most unsightly appearance.

Large nodules can usually be dispersed by applying a shearing force, as in pumping or stirring, or alternatively, coarse filtration can be employed to remove the lumps without too much loss of product viscosity. Smaller particles are, of course, more of a problem, because the force required to bring about their disintegration would destroy the yoghurt coagulum as well. This latter point implies that the nodules have a definite structure, a view supported by Cooper *et al* [3] who showed that the nodules have a higher protein and total solids content than the surrounding yoghurt. It is also of interest that the same authors could find no evidence of aggregates of bacteria within the nodules, so that the proposal that *Lactobacillus bulgaricus* and *Streptococcus thermophilus* act as 'nuclei' during nodule formation cannot be substantiated.

A number of other workers have also examined the nature and/or origin of nodules, and their results can be summarised as follows:-

Suggested causes of nodulation:

i) Weak starter activity;
ii) Poor dispersal of culture;
iii) Variations in incubation temperature;
iv) Inhibitory substances in the milk;
v) Disturbance of the coagulum before it has cooled;
vi) Variable composition of the raw materials, coinciding with seasonal variations in the number of observed faults in yoghurt.

After – Aparicio [4], Casalis [5], Davis [1], Kalab *et al*, [6].

The only conclusion that can be drawn from this assortment of ideas is that confusion is still rife, but two distinct threads can, nevertheless, be discerned, namely:

a) that the quality of the raw materials may be important; and
b) that deficiencies in process control could be equally responsible for causing the defect.

It was decided, therefore, that these two proposals merited more detailed examination, and hence the aim of this present study was to:

i) examine further the nature of the nodules, with the particular view of confirming that rogue activity of the starter culture was not stimulating their formation;
ii) investigate the extent to which variations in process conditions could lead to the production of an uneven coagulum.

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The structure of the nodules

A number of samples of yoghurt showing nodulation were collected, together with some experimental materials (see later), and a random batch of individual particles was extracted from each carton. One characteristic feature of these nodules was the ease with which they could be washed out from the yoghurt by diluting it with distilled water, so confirming their identity as discrete entities. Even quite large 'lumps' rapidly disintegrated to expose a hard, central origin, and the impression emerged that the formation of the central core was the phenomenon of real concern. It was, therefore, these regions that were examined in more detail, for it was hoped that such an appraisal might confirm some of the data presented by Cooper *et al* [3].

The examination was carried out under a scanning electron microscope, and preparation of the materials involved:

1) selecting an isolated, washed nodule;
2) mounting it on a brass 'stud' for insertion into the microscope;
3) freeze-drying the material;
4) fracturing one surface of the nodule to reveal the internal structure;
5) coating the surfaces with platinum;
6) examination of the outer surface and the fracture face at magnifications up to X 3000.

The view of the entire nodule (Figure 1) confirms the earlier impression that the granules have a definite compact structure that is congruous with their existence as discrete particles within the yoghurt coagulum, and the smooth convolutions of the outer surface of the nodules (Figure 2) also emphasises the lack of connection between the casein of the granules and the micelles of the suspending medium. The failure of normal processing techniques to disrupt the intruding particles is also understandable, because ready disintegration would imply a fragility of structure that clearly does not exist. This point was also borne out by examination of the fracture faces, because although the basic arrangement of the casein micelles is similar to that in normal yoghurt, the organisation appears to have become more dense (Figure 3).

Such a conclusion would be in keeping with the finding that the solids-not-fat (SNF) content of the nodules is higher than that of normal yoghurt, and it is also noticeable that the examination produced no evidence of any agglomerations of bacteria.

Obviously the number of nodules that was investigated in this way was severely limited, but present evidence does suggest that there is no reason to suspect abnormal starter activity as being a likely cause of nodulation. If this view is correct, then it becomes necessary to seek other explanations for the occurrence of these irregular aggregations of the casein.

Variations in raw material composition

The majority of manufacturers probably employ the system whereby liquid skim-milk is fortified with milk powder to provide the desired level of total solids in the process milk, and both materials are, within limits, subject to changes in composition. The extent to which skim-milk powders vary from plant to plant, or with season and/or geographical location, is a matter for conjecture, but as liquid skim-milk is the major component, it is probable that variations in this fraction would be the most relevant.

The information available on changes in the SNF content of raw milk suggests that, after a low of 8.6% in March, the level rises to around 8.8% in June, and then broadly stabilises until October [7]. The existence of these trends is now widely accepted, but in the present context, two points are perhaps relevant:

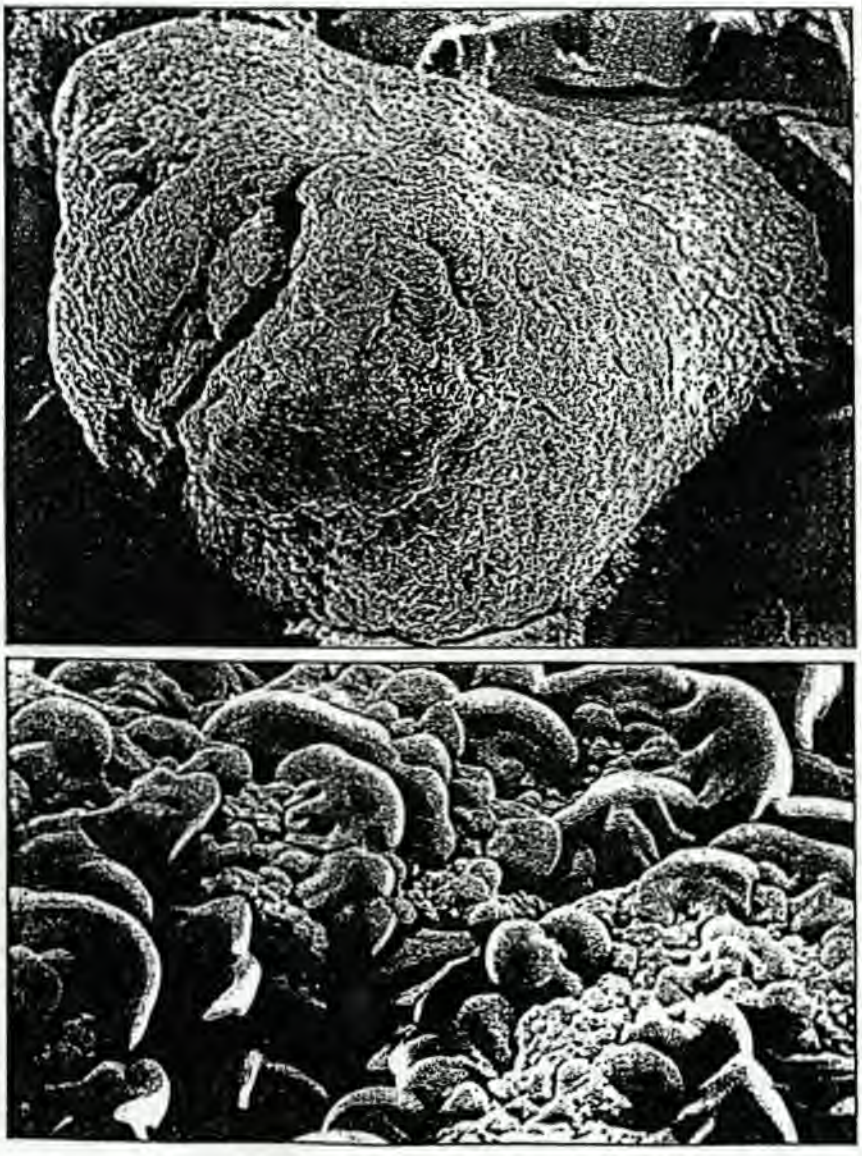
a) the overall changes are small, and subject to variation within the suggested limits; and
b) according to the data of Cooper *et al* (*loc cit*) the incidence of nodulation reaches a maximum during March and April, falls during the summer months, and then 'peaks' again around October.

In other words, even if the figures for SNF are accepted at their face value, heavy nodulation was first observed when the SNF level was at its lowest, and then again during the 'peak time' for SNF. This absence of any obvious correlation between SNF values and the presence or absence of nodules would tend to discredit the proposed association, but for the fact that:

a) the analyses of chemical composition and the evidence of nodulation come from entirely different sources, and it is sheer speculation that the yoghurt milk monitored by Cooper and his colleagues had a 'typical' level of SNF;
b) the figures for SNF make no allowance for changes in the level of individual components. Thus, the calcium content of milk is reported to be higher in winter than in summer [8], and if peak levels occurred in March and October, then it is not inconceivable that the normal acid coagulation of the casein could be disturbed;
c) finally, of course, the whole concept of 'typical' values for SNF may be in question, because while a low level in March and a high one in October may be the normal pattern, Jack *et al* [9] found quite a different position in milk samples taken in California during 1949. In this latter case, the spring and autumn values were almost iden-

Figure 1. A typical 'granule' or 'nodule' extracted from yoghurt; the compact nature of the structure is clearly evident. (X 35)

Figure 2. Surface view of a nodule that emphasises the lack of connection between the particles and the suspending coagulum (X 750); this extreme compaction may not, however, be typical.



Treatment	Frequency of granules CH_1	$Boll_1$
Incubation at 35°C	—	—
Incubation at 42°C	—	—
Incubation at 45°C	+++	—
Incubation at 50°C	+++	++
Stirring at 42°C	+++	+
Stirring at 20°C	++	—
Disturbance of coagulum	+++	—
Slow acid development	+	—

Code:
+++ – abundant + – occasional
++ – frequent — – non detectable

Table 1. Effect of processing conditions on the occurrence of nodulation.

tically high, so that if high levels of SNF were associated with the onset of nodulation, then the data from California would provide valuable support.

Overall, therefore, the conclusion must be that even if the occurrence of 'lumpiness' is seasonal, there is no real indication of which other variable might be linked with the onset of the problem. This rather negative conclusion will not, of course, end the speculation, but it is worth mentioning that, in many instances, nodulation occurs on an entirely 'occasional' basis throughout the year. This random incidence suggests a more prosaic cause, and it was decided, therefore, to examine the extent to which nodulation could be induced by altering process conditions.

Some causes of nodulation

Spray-dried skim-milk (medium heat) was reconstituted to provide a standard raw material at 16% total solids, and the control yoghurt was made by the procedure outlined in Figure 4.

The end-product had a smooth, even consistency, but providing an objective record of this state proved to be something of a problem. The most reasonable solution was simply to spread the yoghurt over a black tile, and then to photograph the dispersed coagulum (see Figure 5). Despite the limitations of this technique, it did provide a means of comparing the consistency of different yoghurts, and of assessing, where applicable, the extent of nodulation.

Once it had been established that the standard procedure could give rise to an acceptable yoghurt, it was possible to determine whether changes in process conditions could cause yoghurt coagulum to become granular, and the following factors were selected for examination:

i) *Incubation temperature*
This examination involved producing yoghurt employing the standard procedure, but with 35°C, 42°C, 45°C and 50°C as the incubation temperatures. In each case, the duration of the fermentation was adjusted to give a final acidity of 0.96–0.98% lactic acid prior to cooling.

ii) *Stirring at 20°C or above*
In this case, yoghurt was made in the normal manner, but was then gently stirred either immediately after incubation (42°C), or after cooling to 20°C. The stirred products were then dispensed into the retail containers for final cooling.

iii) *Disturbance during incubation*
Although care was taken to avoid any visible 'breaks' appearing in the coagulum, the fermentation vessel was shaken at hourly intervals during incubation.

iv) *Choice of starter culture*
Each series of experiments was carried out employing –

a) a culture designated CH$_1$ (Chr. Hansen's Ltd) and

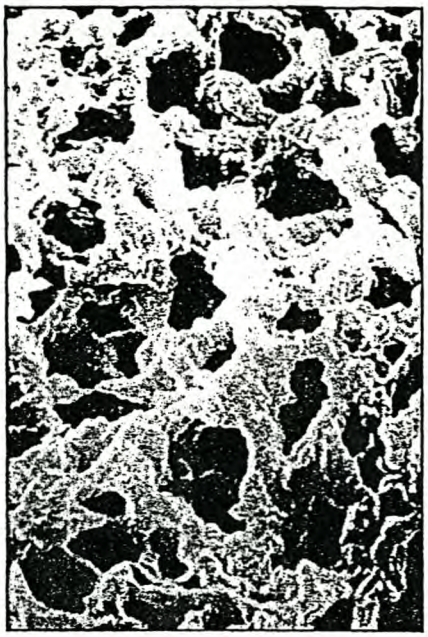


Figure 3. (a) Coagulum of a normal yoghurt sample; (b) Interior of a nodule exposed by fracturing a freeze-dried specimen. (X 2000)

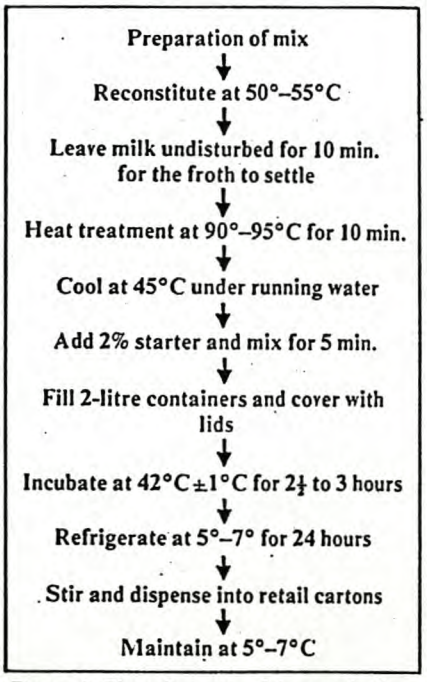


Figure 4. Flow diagram of the standard procedure for the production of yoghurt.

Figure 5. Natural yoghurt spread over the surface of a black tile to reveal the even nature of the coagulum; for method of production see Figure 4.

b) a culture designated Boll$_1$ (Chr. Hansen's Ltd).

In each case, the inoculum was a liquid culture containing *Str thermophilus* and *Lac bulgaricus* in the ratio of 1:1. The inoculation rate was standardised at 2% v/v, except in one trial, when a 0.5% rate was used to assess the effect of slow acid development on the coagulum.

The results of the various trials are summarised in Table 1, and it is clear that it is comparatively easy to induce formation of granules. What is equally evident is that the changes from the standard procedure are not dramatic. Thus, a slight elevation of the temperature (Figure 6) or stirring prior to complete cooling (Figure 7) introduced a totally unacceptable level of nodulation, and furthermore, the magnitude of some of these alterations in process conditions suggests that they could easily be duplicated at plant level.

What is encouraging, however, from the data in Table 1 is that a change in starter culture can ameliorate the situation considerably, although there was absolutely no evidence to suggest that the bacteria in the 'susceptible' yoghurt milk (ie containing

culture CH_1) were in any way responsible for initiating granule formation. Indeed, it is possible (as shown in Figure 8) to cause granule formation in process milk simply by the addition of lactic acid, and hence any criticism of the bacterial cultures *per se* cannot be sustained.

What seems more likely is that the increased firmness imparted to the yoghurt by the so-called 'viscous cultures' (eg $Boll_3$), renders the coagulum resistant to thermal or physical 'shocks' which the more delicate gel formed by CH_1 cannot withstand. If this view is correct, it would imply that a manufacturer of a stirred, fruit yoghurt prone to nodulation might do well to consider a change of culture. Obviously such a move should run concurrently with improvements in process control, but unless the delicate flavour associated with CH_1-type cultures is essential, cf for natural yoghurt, then the switch to a more 'viscous culture' may at least serve to mask any imperfections of manufacture.

It would be irresponsible on the basis of this brief examination of the problem of nodulation in yoghurt to be too dogmatic about its cause, but certain tentative conclusions might bear repetition, namely:-

i) evidence that nodulation occurs mainly at certain times of the year is extremely scant, and indeed a chemical or physicochemical basis for the proposed seasonality has yet to be defined;

ii) the granules, which appear to be compressed aggregates of casein, can be readily induced, on an experimental basis, by deviation from the conditions normally employed for yoghurt production, and it is likely that similar alterations at plant level could be responsible for the formation of nodules in commercial products;

iii) although the starter bacteria have been exonerated as casual agents, the fragile coagulum produced by some strains does appear more prone to faults than the product of the so-called 'viscous' strains. In practical terms, this difference means that a manufacturer may be able to minimize the risk of a faulty product by selection of a more appropriate starter culture.

Acknowledgment

The author gratefully acknowledges the assistance of Dr Maria Cadena throughout the course of this work, and the collaboration of Dr J. R. Burnett in obtaining the photographs of the nodules.

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Figure 6 (a) Natural yoghurt prepared by the standard procedure but with the incubation temperature elevated to 45°C (b) Above right: As above, but with temperature at 50°C.

Figure 7. (a) Natural yoghurt prepared by the standard procedure, but with the coagulum being stirred/dispensed immediately after incubation, ie around 40°C. (b) As above, but with yoghurt cooled to 20°C prior to agitation.

Figure 8. Skim-milk (16% TS) acidified to around 1% lactic acid by the direct addition of lactic acid; note granule formation in the absence of starter bacteria.

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YOGHURT

SCIENCE AND TECHNOLOGY

Second edition

A.Y.Tamime and R.K.Robinson

Yoghurt: science and technology is a standard work in its field for both industry professionals and those involved in applied research. Because manufacture is still, essentially a natural biological process, it remains difficult to control the quality of the final product. Such control depends on a thorough understanding of the nature of yoghurt and both the biochemical changes and process technologies involved in production. *Yoghurt: science* and *technology* provides just such an understanding.

Since the last edition the industry has been transformed by the introduction of mild-tasting 'bio-yoghurts', changing both consumer markets and manufacturing practices. The new edition has been comprehensively revised and expanded to take on board this and other major changes in the industry, for example the production of strained yoghurt by ultrafiltration and the latest advances in mechanisation and automation.

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Dairy products - *the real risks*

Reports of poisoning or infection from dairy products are impressively low, at least for developed countries. But dangers still exist, and Turkan Keceli and Richard K Robinson from the Department of Food Science and Technology, University of Reading, highlight some of the recent product/pathogen associations

It is inevitable that milk from any domestic animal should be prone to contamination by pathogenic bacteria (11). It is important, therefore, that consumers should be aware of the risks involved. It is assumed that herds supplying such milk will be free from *Mycobacterium* or *Brucella* but, unfortunately, the cost of monitoring herds means that lapses could occur (32).

Similarly, there is evidence that *Campylobacter jejuni* and *Coxiella burnetii* can be excreted direct into the milk of apparently healthy dairy cows (50) so that, even if a milking parlour is immaculate, the transmission of pathogens can still occur. Faecal contamination remains, of course, the most blatant risk, and the many studies of the microflora of raw milk (19,1,9,14) have always reached the same conclusions: (a) do not drink raw milk; and (b) do not use milk in food products unless it receives a preliminary heat treatment.

It is generally agreed – despite some queries concerning *Listeria monocytogenes* – that the pasteurisation of milk at 71.7°C for 15 seconds (or an equivalent treatment) will eradicate all non-sporing bacterial pathogens from milk; the presence of protozoans like *Cryptosporidium* does, of course, necessitate a more severe treatment. However, despite the confidence that the system inspires, many of the milk-associated cases of salmonellosis reported in the UK between 1950 and 1980 could be traced to the consumption of defectively pasteurised cow's milk, or milk that had been contaminated after pasteurisation (19); more recently, significant disease outbreaks resulting from pasteurised milk contaminated with *Salmonella typhimurium*, *Salmonella braenderup* and *Campylobacter jejuni* have been reported (33,29).

L monocytogenes is also an occasional contaminant of pasteurised milk within the EEC (13,55). Similarly in the USA, pasteurised milk has acted as a carrier for *S typhimurium* and *L monocytogenes* (18). *Yersinia enterocolitica* can be transmitted through pasteurised milk as well (24), and problems with recovery (23) suggest that the true level of occurrence could be underestimated.

An additional concern is the growing exposure of cattle to antibiotics. Thus, a study in Mexico (16) found that 67% of retail samples of pasteurised milk contained faecal coliforms with multiple drug-resistance, while penicillin-resistant strains of *Salmonella* and *Staphylococcus aureus* were also detected. A similar picture emerged in Trinidad during a study of raw milk (3) and, while these organisms should not be present in retail milk anyway, the ease with which this pool of drug-resistant organisms is entering the food-chain would appear to merit further attention.

All these cases have been linked with (a) poor plant design, ie no 'fail safe' system in the event of operator error; (b) poor maintenance that has failed to detect 'pin holes' allowing raw milk to contaminate heat treated product; and (c) inadequate quality assurance procedures. While Hazard Analysis of Critical Control Points may reduce the risk of a problem arising, an examination of a representative sample of retail items remains essential. This view is confirmed, to some extent, by the data in Table 2, which indicate that even the examination of random (unofficial) samples can be extremely successful in detecting faults in market milk.

It has been proposed that pasteurised milk should have a total colony count of less than 100,000 colony-forming units (cfu) ml^{-1} after 5 days at 6°C starting from an initial count of <30,000 cfu ml^{-1} (49). The reality of these standards was established in a survey of UK market milk which showed that 4% of samples had total counts < 1,000 cfu ml^{-1}, 85% in the range 1,000-100,000 cfu ml^{-1} and only 11% were above the ceiling of 100,000 cfu ml^{-1}. More recently, it was found that the quality of pasteurised milk tended to improve with scale of operation, suggesting, perhaps, that quality control in some small to medium-sized factories is in need of improvement (13).

Non-fermented milk products

Cream is just as susceptible to spoilage as the milk from which it was

Table 1. Some of the important outbreaks of food-born disease in which cheese was implicated as the cause

Cheese	Organism	Country	Year
Camembert	*Salmonella bareilly*	USA	1953
Camembert	*Escherichia coli* (enteropathogenic)	USA	1971
Brie	*Escherichia coli* (enteropathogenic)	USA	1971
St. Marcellin	*Shigella sonnei*	France	1964
Fresh White Cheese	*Brucella abortus*	Italy	1972
Queso Blanco	*Listeria monocytogenes*	USA	1985
Cheddar	*Staphylococcus aureus*	USA	1968
Cheddar	*Staphylococcus aureus*	UK	1960
Cheddar	*Staphylococcus typhimurium*	Canada	1984
Cheddar	*Staphylococcus heidelberg*	USA	1976

separated, and the process of separation can often lead to a concentration of spores into the fat layer. During further heat treatments, the fat can, to a degree, insulate the bacterial cells, and this fact is recognised in the USA where dairy products with more than 10% fat have to be pasteurised at 74.4°C for 15 seconds. The protective effect of fat could explain the high counts sometimes recorded for double cream (49), although the temptation to over-pasteurise could be equally important. Thus, heat-treatments above 72-74°C for 15 seconds offer little advantage (12), as the heat-shock applied to the spores leads to more rapid germination and spoilage; a point relevant also with respect to pathogens, because the flora of spoiled cream is often dominated by strains of *Bacillus cereus*.

Butter should be a 'safe' product for the consumer, in that: (a) the majority of bacteria will be removed with the buttermilk: and (b) in well-made butter, the majority of the water droplets should be below 10 µm in diameter, so providing neither space nor nutrients for bacterial growth. Even the moulds often associated with the spoilage of butter, eg *Alternaria* or *Cladosporium*, are not known to produce mycotoxins, and hence only gross contamination from contact surfaces during reworking or packing is likely to cause serious problems.

With milk powders, spray-drying involves the almost instantaneous drying of droplets of milk and their agglomeration into small granules. Once formed, any bacteria trapped in the granule are protected from the hot air within the chamber, so that even outlet temperatures over 100°C may have little effect on either vegetative cells or endospores. For example, both *S typhimurium* and *Salmonella thompson* have been shown to survive normal spray-drying and storage (39). However, because the milk will have been heat-treated prior to evaporation and drying, non-spore-forming pathogens should not be present in the drying chamber.

The source of most problems linked with milk powders have been found to have a focus around the packaging area, and various species of *Salmonella* are the most serious pathogens likely to be associated with milk powders. *S newbrunswick* was responsible for an outbreak of salmonellosis in the USA (15), where a faulty sanitising plant led to the contamination. *S agona* and *S typhymurium* have also been implicated with incidents in the USA, and hence it is not surprising that major dairy countries like Australia insist on end-product analysis of all milk powders for salmonellae. The expected standard is 'absent in 200g' although 'absent in 500g' is often employed as an operational target (20).

The contamination of milk powders with coagulase-positive strains of Staph aureus have led to a number of reported incidents of food-poisoning, so that the normal standard is now 'absent in 25g' of powder; the same standard is applied to faecal coliforms. Lovell (41) reports that *Yersinia enterocolitica*, as well as various species of *Enterococcus*, have been linked with disease outbreaks associated with foods that incorporated milk powder. Although there was, in these cases, no evidence that the milk powders were directly involved, it reinforces the need for powder manufacturers to operate to strict standards of hygiene – if only to avoid being open to criticism.

The presence of any of these vegetative pathogens implies post-pasteurisation contamination, but spore-formers are more likely to enter with the raw milk. The spores of *B cereus* were found in dried milks in Egypt in numbers ranging between 10 and 10 x 10^6/g (27), and the use of such powders could lead to incidents of food-poisoning. *Clostridium perfringens* is another pathogenic spore former that has been found in milk powders, particularly when the powder has been packed under vacuum or in tightly sealed, polythene bags. However, it has been recommended that the polythene liners of bags for SMP should have 'pin-holes' to allow for ease of stacking (17), and this should ensure that bags are sufficiently aerobic to encourage a decline in numbers of clostridia.

The high total solids of most ice cream mixes – typically 10% fat, 10% milk solids-non-fat, 14% sugar and 0.5% stabiliser – means that the pasteurisation temperature/time regime is changed to 71.1°C for at least 10 minutes (or equivalent) to ensure the elimination of vegetative pathogens. In addition, National Regulations will usually stipulate maximum holding temperatures and times before and after heat treatment, so that contaminants should have little opportunity for growth. Given the application of Good Manufacturing Practice, it should be possible to meet the targets suggested in (20) of total colony count: <50,000 cfu/g, coliforms <10 cfu/g, faecal coliforms and *Staph aureus* 'absent in 1 g' and salmonellae 'absent in 25g' with 'absent in 100g' as a desirable target.

Large-scale manufacturers can usually achieve these specifications, but two sectors merit close attention. The first is occupied by small manufacturers in Asia, the Middle East and elsewhere, for there are reports from Egypt (46), India (59), Iran (54) and Turkey (7) of locally-made ice creams that were totally unacceptable. Probably a combination of low-grade ingredients, poor hygiene and process control contributed to the low microbiological quality and, although coagulase-positive staphylococci and *E coli* were the only pathogens identified in any of the samples, the potential dangers for the consumer are self-evident. In addition, pathogens can survive at appreciable levels in ice cream (4). In Europe, small producers can encounter problems with ingredients as well, and an outbreak of *Salmonella enteritidis* PT4 infection was linked with the use of raw eggs in an exotic recipe (48).

Retailing is the other sector that deserves further scrutiny. Thus, the practice of 'scooping' ice cream from a carton and serving it on a cone can lead to problems from poor hygiene, and the microbial quality of the so-called 'soft serve' ice creams can be equally suspect (56). Of over 800 samples of ice cream tested in Louisiana, nearly 40% had total counts in excess of 50,000 cfu/g, and over 50% had coliform counts >10 cfu/g.

Table 2. A record of 'official' and 'informal' samples of milk and milk products examined by enforcement officers in the UK during 1995; the figures in brackets indicate the number of incidents that lead to prosecution.

Product	Official Samples		Informal Samples	
	Total	Unsatisfactory	Total	Unsatisfactory
Market Milk	7,777	1,277 (2)	16,740	2,961
Cheese	976	126 (0)	1,188	228
Butter	121	10 (0)	54	6
Others	1,533	202 (3)	3,631	516

(After: 33)

Fermented milk products

Yogurt and similar fermented milks should be entirely safe for the consumer because, with a pH in the range of 3.8-4.2, vegetative pathogens like *L monocytogenes*, *E coli*, *Klebsiella*

pneumoniae, *Y enterocolitica* and *Salmonella* will be killed even before the retail product leaves the factory (5,22). However, poor process control or low starter activity can change the picture dramatically, and yogurt was the source of an outbreak of *E coli* (VTEC) 0157 infection in the UK (47).

A similar problem could arise with products manufactured with probiotic cultures, for the pH of 'bio-yogurts' is deliberately held around 4.6. This figure is chosen because (a) many consumers enjoy the milk flavour; and (b) certain strains of the desired organisms, such as *Lactobacillus acidophilus*, are acid-sensitive, and will decline below the therapeutic minimum count of 1 x 10^6 cfu/g of product on prolonged exposure to acidic conditions. However, pathogens like *Staph aureus* can survive under conditions of medium acidity, and hence high standards of plant hygiene are essential. It may be helpful that a number of lactic acid bacteria release bacteriostatic/bactericidal metabolites (57) but, with the exception of nisin, there appears to be some doubt as to how effective such materials are in food systems.

Cheese risks

In considering the potential risks posed by the consumption of cheese, the important division between soft, fresh cheeses (moisture content >60%) and hard, grating cheeses like Parmesan (moisture content <35%) is obviously going to be crucial with respect to microbial activity, and the available data bear this distinction out; Italian cheeses of the Romano-type, for example, never appear to have been cited as causes of a disease outbreak.

Consequently, it is the unripened, high moisture cheeses, such as queso blanco or cottage cheese (31), or the externally mould-ripened like Brie or Camembert (30) that tend to give most cause for concern with regard to *Listeria* or other pathogens (see Table 1). The tendency for small manufacturers to use raw milk is the source of many problems, and an outbreak of infections by *Salmonella dublin* following consumption of a soft cheese is a case in point (43); a disease outbreak arising from *Salmonella enteritica* serotype *paratyphi* B in France had a similar origin (53).

L monocytogenes has been isolated also from soft, unripened cheeses (55,25), and counts in a few samples exceeded 1,000 cfu/g; it is relevant that growth of *Listeria* during transfers of cheese within the retail chain have been reported (45). This growth potential suggest that standards of 'absent in 25g' should be set for soft cheeses (5 samples/batch), with a more lenient count for hard cheeses; Pether *et al* (51) suggest 'absent in 25g' for hard cheese made from pasteurised milk, with the proviso that one of the five samples may fall outside this limit. A further suggestion is that a figure of <20 cfu/g would be acceptable for Cheddar cheese at the end of manufacture, on the grounds that the numbers will decline during storage; counts around this level should not cause infection; and the specifications for other ready-to-eat foods have been set at <100 cfu/g (42). However, such guidelines should acknowledge that all isolates of *L monocytogenes* are potentially pathogenic, because the absence of a hospital record citing a specific serotype does not imply that it cannot cause infection.

White brined cheeses tend to harbour rather low counts of *Listeria* spp (21) but other pathogens, such as *S typhimurium* and *Staph aureus*, tend to die out rapidly (2,44). In queso fresco, the growth of *E coli* 0157 was inhibited below 10°C (37), but Little and Knochel showed that *Y enterolitica* could grow on Brie at 4°C (40).

The situation with hard and semi-hard cheeses is less straightforward for, while there have been some major incidents associated with hard cheeses, it has been shown (8) that when *Aeromonas hydrophila*, *C jejuni*, *E coli*, *Pseudomonas aeruginosa*, *S typhimurium*, *Staph aureus* and *Y enterocolitica* were added to the milks for making test hard cheeses (Emmental and Gruyère) and semi-hard cheese (Tilsit and Appenzeller), only *L monocytogenes* survived a normal ripening period of 45-55 days. However, no growth of Listeria was found, and no staphylococcal toxins were detected either.

It has been noted that the microbiological quality of many American hard/semi-hard cheeses has improved over the last twenty years (10,60). The almost universal use in the USA of a heat-treatment capable of eliminating pathogens from cheese milk may be important in this context, for in Spain 51% of samples of Cebrero cheese (raw milk) contained high counts of *E coli* and 20% had excessive levels of *Staph aureus* (52).

Such problems with hard cheeses made from raw milk are probably common than the literature suggests, but factors other than pasteurisation may be contributing to the rising standards observed in the USA, namely:

- Improved standards of hygiene: thus, while the milks for Bafut and Tulum cheeses are pasteurised, samples of these semi-hard cheeses have been found from Cameroon and Turkey that would pose a considerable risk to human health (36,38). In both cases, poor process control was considered the likely source of contamination.
- Improved control of starter cultures: Jervis (33) established that both salmonellae and staphylococci will grow in fresh Cheddar cheese curd at pH 5.6, while no growth, followed by a decline during maturation, is observed with normal curds at pH 5.0. This conclusion implies that the reported incidents of *Salmonella* contamination of Cheddar cheese could be directly attributed to starter failure. Over the last ten years, such failures have been almost eliminated through: (i) the introduction of culture rotation to avoid problems with phage; (ii) antibiotic testing of all process milks to avoid starter inhibition; (iii) the use of concentrated direct-to-vat cultures with guaranteed levels of activity; and (iv) improved control over the stages of manufacture, such as salting, that can adversely affect the level of acid production achieved at the time of pressing.

In addition, specific characteristics of hard cheeses may be important in controlling pathogens, for example: (a) a number of sub-optimal conditions with respect to the growth of certain pathogens may limit their development (44); and (b) chemical components in the cheese may be having an antimicrobial effect. Thus, aside from bacteriocins that may be released by the starter culture, the hard/semi-hard varieties of cheese will contain both short-chain free fatty acids, such as butyric, caprylic and capric, as well as medium-chain acids like linolenic and linoleic. These acids, along with various monoglycerides, are inhibitory, to varying degrees, to bacteria and, given that their concentrations will build-up during maturation as a result of lipolysis of milk fat, they could well contribute to the safety of matured cheese (58,35).

Conclusions

There appears to be an encouraging trend with respect of the safety of dairy products, in that major incidents of milk-borne disease with origins in large-scale factories are becoming less frequent. Conversely, it is clear that the careless handling of milk, together with the irrational desire to use raw milk, remains a serious threat to consumers, for as shown in Table 2, the number of microbiologically unsatisfactory samples of dairy produce discovered in the UK during 1995 is not encouraging. Clearly a policy of 'zero tolerance' for even trivial faults has much to commend it, and it is to be hoped that more small dairy companies will move in this direction. ■

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PAPER

The direct enumeration of *Listeria monocytogenes* in a food with a low background microflora

V.S. Gohil,* M.A. Ahmed,* R. Davies† and R.K. Robinson†‡

Concentrated yoghurt (labneh) with an inherently low count of non-lactic acid bacteria was inoculated with various levels of Listeria monocytogenes, *and direct plating onto Listeria Selective Agar or PALCAM Agar proved effective for monitoring high counts. At levels of < 10 cfu/g, an MPN count using UVM I/Fraser Broth was more reliable, and a 'presumptive' count, taking the colour change in Fraser Broth from yellow to black as being 'positive', had a failure rate of < 2%. Given that such low counts are below the likely infective dose for humans, it is suggested that the direct MPN count could be employed for routine quality control examinations.*

Keywords: labneh, *Listeria*, enumeration

INTRODUCTION

It has been suggested that ready-to-eat foods should be sold against a specification of 'absent in 25 g' covering *L. monocytogenes* (Lund, 1993), and this proposal raises the question as to how samples of a retail product should be examined, on a routine basis, for low levels of contamination. Obviously the standard methods recommended by the FDA (Lovett *et al.*, 1987), USDA (McLain and Lee, 1989) and IDF (1990) are well established, but these procedures can be demanding for a small laboratory. In particular, each method involves selective enrichment for 48 hours, followed by plating onto selective agar and incubation for a further 48 hours; where necessary, confirmatory tests for the presence of *L. monocytogenes* may be needed as well.

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More rapid methods are available, e.g. the ELISA reaction (Anon, 1991) and similar approaches (Roberts, 1994), for the detection of *Listeria* sp., or API strips (Anon, 1994a) for identification of the species, but the cost of such techniques can prove daunting in a routine quality control situation.

The rationale behind the standard methods is the detection of *Listeria* against a complex background of other genera but, if the background count is low and/or restricted with respect to the types of contaminant – a situation that might well arise with respect to end-product examination in a factory, then a simplified approach might become a feasible option. In the United Arab Emirates, one product that should meet these criteria is labneh, a concentrated yoghurt fermented with a starter culture consisting of *Lactobacillus delbrueckii* sub-sp. *bulgaricus* and *Streptococcus thermophilus*. The conditions of manufacture on a commercial scale ensure that bacterial contamination is kept to a minimum (Robinson and Tamime, 1993), and hence the range of genera competing on the media

Table 1 Results of the microbiological and chemical analyses of the labneh used for 'spiking' with *L. monocytogenes*

	Microbiological Analysis	Chemical Analysis	
Test		Test	
Aerobic plate count	< 100/g	Fat	10%
Coliforms	< 10/g	Total solids	27%
Faecal coliforms	Not detected in 1 g	pH	3.8
Staph. aureus	< 10/g		
Yeasts and moulds	< 10/g		
Listeria sp.	Not detected in 25 g		

employed for the selective enumeration of *Listeria* should be mainly limited to those of starter origin. Consequently, it was decided to:

(i) obtain commercial batches of labneh from one source;
(ii) inoculate different portions with estimated counts of *L. monocytogenes* ranging from < 10 to > 1000 colony-forming units (cfu)/g of labneh;
(iii) enumerate *L. monocytogenes* in the product by a standard plate count technique using Listeria Selective Agar (Oxford Formulation) and PALCAM Agar (Anon, 1994b); and
(iv) compare the recovery of *Listeria* on the above media with counts estimated by a Most Probable Number (MPN) procedure using UVM I/Fraser Broth (Fraser and Sperber, 1988) and Listeria Enrichment Broth (Anon, 1994b).

MATERIALS AND METHODS

Bulk samples of chilled, unsalted labneh collected from a local dairy on the day of production in suitable sterile containers and, with the temperature maintained below 4°C, were delivered to the laboratory for immediate analysis. At the laboratory, representative sub-samples were subjected to chemical analyses for pH, fat and moisture employing methods based on those described by Egan *et al.*, (1981).

In addition, the samples were examined for total bacterial load – non-lactic acid bacteria (aerobic plate count on Plate Count Agar at 35°C for 48 hours); coliform count (Violet Red Bile Agar at 35°C for 24 hours, followed by confirmation in Brilliant Green Bile Broth at 35°C for 24–48 hours); presence of faecal coliforms (MacConkey Broth at 35°C for 24–48 hours, followed by confirmation in Brilliant Green Bile Broth at 44.5°C in a water bath for 24–48 hours); *Staphylococcus aureus* (Baird Parker Medium at 35°C for 48 hours, followed by confirmation of black colonies with or without an opaque zone by the coagulase test) (Anon, 1994b; Richardson, 1985).

A specific examination for any naturally occurring *Listeria* involved weighing 25 g of labneh into a sterile Stomacher bag, and adding 225 ml of Listeria Enrichment Broth (LEB) (Lovett *et al.*, 1987). The contents of the bag were blended in a Stomacher (Type 400) for 30 seconds, and incubated at 35°C for 48 hours. A loopful was then streaked onto Listeria Selective Agar (LSA) and incubated at 35°C for 48 hours (Curtis *et al.*, 1989).

The results of the chemical and microbiological analyses are shown in *Table 1* and, although the level of total solids was above average for labneh, the quality of the experimental material was broadly similar to brands available elsewhere (Robinson and Tamime, 1993). It was evident also that the numbers of contaminant micro-organisms were at levels too low to have any decisive influence on the behaviour of the *Listeria* to be inoculated later.

THE TEST ORGANISM

The organism chosen for the tests was *L. monocytogenes* NCTC 10888, and the stock culture was maintained on Tryptone Soya Yeast Extract Agar (TSYEA) slants incubated at 35°C for 24 hours and stored at 4°C for up to 5 days. At the start of the programme, identification to genus and species level was confirmed using the procedures described below, and the same tests were applied to a random selection of typical colonies isolated during the examination of the inoculated labneh.

CONFIRMATION TO GENERIC LEVEL

Reaction to Gram's Stain

A smear of the organism was prepared by placing two drops of sterile distilled water onto a clean slide, and adding a small amount of material from a colony isolated on TSYEA. The smear was heat-fixed and stained in the conventional manner (Harrigan and McCance, 1976). *Listeria* sp. appear as Gram-positive, short bacilli – often blue to violet in colour (Seeliger and Jones, 1986).

Motility Test

Typical colonies on TSYEA were inoculated into tubes of Brain Heart Infusion Broth (BHI), and the tubes were incubated overnight at ambient temperature (20–25°C). Motility was observed under the light microscope by the Hanging Drop Method (Harrigan and McCance, 1976). All *Listeria* appear motile when incubated at ambient temperature, and show a characteristic tumbling or rotating motility.

Catalase Reaction

Drops of H_2O_2 (3%) were placed onto clean slides and, with a wire loop, portions of the same colonies used for the above tests were picked from the TSYEA agar and stirred into individual drops of hydrogen peroxide. The test was considered positive when immediate bubble formation or effervescence was produced. All *Listeria* are catalase-positive.

Once any given colony had been confirmed as a probable *Listeria*, the following tests were then carried out for identification of the species.

(i) *Haemolysis.* The isolate was streaked onto Columbia Agar plus Horse Blood (5%) and incubated at 35°C for 24–48 hours. The plates were examined for haemolysis by holding the plate at an angle of 90° and viewing from the base. *L. monocytogenes* produces the narrow zone of beta-haemolysis.

(ii) *The CAMP Test.* Beta-haemolytic strains of *Staphylococcus aureus* (NCTC 7428) and *Rhodococcus equi* (NCTC 1621) were streaked as two separate lines onto Nutrient Agar overlayed with Nutrient Agar plus Sheep's Blood (5%). An isolate of *Listeria* was streaked at right angles between the two lines without quite touching them. After 24 and 48 hours incubation at 35°C, the plates were examined for haemolysis in the 'zone of influence' between the competing cultures. Haemolysis by *L. monocytogenes* is enhanced only in the vicinity of *Staph. aureus.*

(iii) *Identification of Species by API*-Listeria. In addition to the above tests, biochemical identification was carried out using API *Listeria* Strips (biomérieux UK Limited, Basingstoke, Hampshire RG22 6HY). The reactions produced during the incubation period were revealed either through spontaneous colour reactions, or following the addition of reagents. After incubation for 18–24 hours at 37°C, the reactions were recorded and, using a chart provided by the manufacturer, the identification was obtained using a profile list.

PREPARATION OF THE INOCULUM

A loopful of *L. monocytogenes* from the TSYEA slant was suspended in 0.1% peptone water, and ten-fold serial dilutions were prepared up to 10^{-8}. Duplicate pour plates were prepared from each dilution using TSYEA agar and when the agar had solidified, the plates were inverted and incubated for 48 hours at 35°C. All the dilutions were stored at 4°C.

At the end of incubation period, the mean counts on TSYEA plates with 30–300 colonies were employed to calculate the number of colony-forming units of *L. monocytogenes*/ml of the suspension in question. The volume of any given suspension required to give a specified cell count in labneh could then be calculated; growth of the *Listeria* in the peptone water during storage was assumed to be negligible.

PROCEDURE FOR INOCULATION

Twenty-five grams of labneh were weighed into each of the Stomacher Bags, and 1 ml of the dilution required to give < 10/g was added to each bag; blending in a Stomacher for 60 seconds was established as the minimum time that ensured an even distribution of the inoculum. This procedure was repeated a further four times, so that a range of anticipated cell counts was attained, namely < 10/g (estimated count of 7 cfu/g), < 100/g (estimated counts of 66 and 89 cfu/g), < 1 000/g (estimated count of 304 cfu/g) and > 1 000/g (estimated count of 2 880 cfu/g). The level of < 100 cfu/g was repeated twice because, as a food with a count in this range might be considered 'suspect' (Lund, 1993), it seemed desirable to check the efficiency of enumeration at this level of contamination.

ENUMERATION OF *L. MONOCYTOGENES* IN LABNEH

Spread Plate Method

Once a given sample of labneh had been inoculated, a first dilution (1:10) was prepared by adding 225 ml of 0.1% peptone to a portion of the labneh (25 g), and blending in a Stomacher for 30 seconds; additional ten-fold dilutions were prepared by adding 11 ml of the previous dilution to 99/ml of sterile diluent (Richardson, 1985). Aliquots of 0.1 ml from the two lowest dilutions were transferred onto plates of LSA and PALCAM Agar in duplicate and, after spreading the inoculum with a sterile bent glass rod, the plates were incubated for 48 hours at 35°C.

After incubation, typical colonies on LSA, i.e. those with a sunken centre and surrounded by a black halo, were counted and recorded, and on PALCAM Agar, typical colonies were taken as those that showed a sunken centre with a black zone around the colony against a cherry red background; confirmation of typical colonies to generic level involved Gram staining, the Motility Test and the Catalase reaction.

The number of cfu per gram of labneh was calculated from the mean of a duplicate plate count multiplied by ten times the dilution factor, and the overall means for five samples at each estimated level inoculation are shown in *Table 2.*

Most Probable Number (MPN) Method

The dilutions prepared above were used, and the three lowest dilutions, namely 10^{-1}, 10^{-2} and 10^{-3}, were employed when the estimated cfu/g was less than 1 000; a fourth dilution was included when the addition exceeded 1 000 cfu/g. For each level of anticipated cell count, 1 ml of the three (four) dilutions was transferred into three test-tubes each containing 10 ml of LEB or UVM I, and the tubes were incubated for 48 and 24 hours at 35°C, respectively. After 24 hours, 0.1 ml was transferred from each tube of UVM I to a tube containing 10 ml of Fraser Broth; a further period of incubation for 24 hours at 35°C was completed.

After incubation, all the tubes of LEB and Fraser Broth were streaked onto LSA. The tubes were considered 'positive' if typical colonies grew on the selective medium. The MPN count was determined using the tables cited in Harrigan and McCance (1976), and the

Table 2 Recovery of *L. monocytogenes* by two methods – spread plates on PALCAM Agar and Listeria Selective Agar (LSA) and an MPN procedure using Listeria Enrichment Broth (LEB) and UVM/Fraser Broth; all counts as colony-forming units (cfu)/g of labneh and means of five replicates

Level of inoculum (Estimated count)	Spread plates PALCAM (cfu/g)	LSA	MPN Counts LEB (cfu/g)	UVM I–FB
7 cfu/g	No count	No count	5 ± 3	16 ± 6
66 cfu/g	< 100	< 100	57 ± 39	57 ± 39
89 cfu/g	< 100	< 100	138 ± 105	57 ± 39
304 cfu/g	278 ± 2	300 ± 60	246 ± 92	258 ± 83
2 880 cfu/g	2 728 ± 281	2 300 ± 355	4 242 ± 2 305	3 771 ± 3 052

most probable number of *Listeria* per gram of labneh was obtained by calculation. The results are shown in Table 2.

RESULTS AND DISCUSSION

Since 0.1 ml of the first dilution represented the highest concentration of cells spread onto the agar plates, the minimum number of colony-forming units per gram required to record a meaningful count by this technique was > 100 cfu/g; for this reason, the spread plate technique did not yield any colonies on the selective agars when the level of 'spiking' was < 10 cfu/g. When the anticipated counts were > 10 but < 100 cfu/g, a mean count of one colony from the duplicate plates was recorded in *Table 2* as an estimated count of < 100 cfu/g (Richardson, 1985).

When the samples were 'spiked' with an estimated 304 cfu/g, all the results on PALCAM Agar fell in the range of 250–300 cfu/g, while on LSA, the range of counts was 200–350 cfu/g. Given that the 95% confidence limit for these counts was calculated as ± 73 (Harrigan and McCance, 1976), it might be concluded that, so long as the likely numbers are in excess of 100 cfu/g, either medium could be employed for the direct enumeration of *Listeria*.

This view was confirmed, to some extent, by the results with product 'spiked' with > 1 000 cfu/g of *L. monocytogenes* (see *Table 2*). Thus, recovery from samples 'spiked' with around 2 900 cfu/g gave figures on PALCAM Agar between 2 600 and 2 900 cfu/g, and on LSA between 2 000 and 2 900 cfu/g. Even though some of these counts, especially on LSA, fell outside the limits of 95% confidence by quite a margin, the overall mean on PALCAM was in sufficient agreement with the estimated count as to confirm that direct plating is a reasonable proposition for foods containing high numbers of *Listeria*, but few competing genera.

With the MPN procedure, the pattern of accuracy of 'recovery' was reversed in that, when the sample was 'spiked' with less than 10 cfu/g, the results with either medium were in the range of 4–23 cfu/g; these results were within the range of the 95% confidence limit described in the MPN Tables. However, when 'spiked' with > 10 but < 100 cfu/g, wider variations about the mean began to appear and, above 1 000 cfu/g, even the means became poor indicators of the anticipated values. The main reason for this deviation from the expected result was the extreme variability between replicates but, as the intended application of the MPN technique was with low-count products, no attempt was made to identify the precise cause of the poor repeatability when the counts were above 1 000 cfu/g.

Overall, it was evident that the MPN method worked better at very low levels of contamination (< 10 cfu/g), whereas the spread plate technique was more precise for enumerating higher counts. Both solid media performed well, but the spread of counts seemed lower on PALCAM Agar. In addition, since this study was restricted to examining a food in which the competing microflora was limited, the relative selectivity of the two media was not tested.

Although the broths employed for the MPN Method gave comparable results, the UVM/Fraser Broth route was found to have one distinct advantage, in that the Fraser Broth changed in colour from yellow to black when a 'positive' was recovered. This change meant that presumptive counts could be recorded at the end of 48 hours, as against 96 hours following growth in LEB and plating onto LSA. To confirm the validity of the technique, all colour changes in Fraser Broth were noted before all the presumptive positive/negative tubes were streaked onto LSA for isolation and confirmation of typical colonies of *L. monocytogenes*. The results showed that every tube of Fraser Broth that showed black colouration was confirmed as 'positive', and only in one case – anticipated count < 10 cfu/g – was a presumptive 'negative' tube found to 'positive' on streaking. This figure represented a failure rate of less than 1.7%, and hence it could be that the MPN Method using UVM I/Fraser Broth could be recommended as a presumptive test for *Listeria* in foods with limited microbial loads.

The frequency of 'false' positives, caused by other genera able to hydrolyse aesculin into products that will complex with ferric ammonium citrate, would need to be evaluated for any given food, as would the possible occurrence of aesculin-negative strains of *Listeria*. Nevertheless, the ease of operation suggests that the MPN technique employing UVM I/Fraser Broth merits serious consideration for the routine quality control of processed foods. The more so, perhaps, if it can be established that 'failures' are only observed when the counts for *Listeria* are so low as to pose little risk to consumers (Gilbert, 1992).

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ORIGINAL ARTICLE

Growth and survival of *Listeria monocytogenes* in two traditional foods from the United Arab Emirates

V. S. Gohil[1], M. A. Ahmed[1], R. Davies[2] and R. K. Robinson[2]*

The survival of Listeria monocytogenes *in two foods of Arabic origin, namely labneh and houmos, was studied. Counts of* L. monocytogenes *in excess of 10 000 cfu* g^{-1} *from labneh (pH 3·8) were reduced to zero within 72 h at 4 and 10°C, but increased survival was noted at pH 4·5, especially in unsalted labneh; at 30°C, no organisms could be detected 24 h after inoculation. From a starting count of 27 000 cfu* g^{-1}, L. monocytogenes *was able to survive on houmos throughout the anticipated shelf life (3 days); when houmos was stored at 4 and 10°C under olive oil, the numbers of* Listeria *declined slowly over 3 days. It was concluded that care should be taken to avoid the contamination of houmos with* Listeria *during preparation in the kitchen.*

Introduction

Although a recent survey of retail foods from the United Arab Emirates (UAE) revealed that the incidence of *Listeria* was low (Gohil et al. 1995), the range of products did not include base materials that might be further modified in the kitchen. Thus, uncooked foods into which fresh herbs, spices or other ingredients are blended manually in the home are very popular among the national and expatriate populations of the UAE, and careless handling could present an obvious risk. The high ambient temperatures of the Gulf also fuel concerns about the possibility that a home-made dish could become suspect following cross-contamination with *Listeria*; the often fatal consequences of infection for unborn or neonatal infants has given listeriosis a higher profile than is, perhaps, merited by the number of recorded incidents.

Consequently, the survival of *L. monocytogenes* in two foods, chosen on the basis of their widespread popularity, was followed over their anticipated shelf lives. The selected products were labneh, a milk-based fermented food made by concentrating yoghurt, and houmos, a thick gruel made from chick peas and tahina (sesame) paste. In both cases, the products are often subject to extensive manipulation before serving.

Nature of the products

Labneh is prepared both at home, as well as commercially in the UAE, and the basic yoghurt is produced with a starter culture consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*; after incubation at 42°C to the desired acidity, some manufacturers blend in a quantity of salt. This yoghurt is then poured into

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muslin bags and hung for 48 h at 1–4°C. Gentle mixing to obtain a smooth consistency follows, and the finished labneh is placed in suitable containers. The composition of a typical commercial brand from the UAE: 27% total solids; 10% fat; 9·0% protein; 1·0% salt and pH 3·8 is about average for the product in question (Robinson and Tamime 1993); the anticipated shelf life is 15 days. Obviously the low pH of commercial labneh should be bacteriocidal to *Listeria* but, as batches made in the home are often less acidic, it was felt that the examination was justified.

Houmos is a popular choice as a cold starter for a meal, and is included almost daily in the Arabic diet. It is made from mashed chick peas and sesame paste, to which salt, garlic, lemon and chopped green pepper are added, along with a little olive oil on the top. Different ratios of mashed chick peas and sesame paste are employed to suit individual tastes, but a typical composition might be: 29·4% total solids; 5·0% fat; 5·2% protein; 1·7% ash and pH 5·5. Unlike labneh, the shelf life for fresh houmos is only 1 day, and it is generally not used after 24 h from the time of preparation; houmos stored under olive oil may be held for 2–3 days before consumption.

Materials and Methods

Preparation of the inoculum

L. monocytogenes NCTC 10888 was cultured for 24 h at 35°C on slants of trypticase soya yeast extract agar (TS-YEA) (Anon. 1994) and, after making an initial suspension, 10-fold serial dilutions to 10^{-8} were prepared in peptone water (9 ml). Duplicate aliquots (1 ml) from each of the three highest dilutions (10^{-6}, 10^{-7}, 10^{-8}) were transferred to sterile Petri dishes, and TS-YEA (12–15 ml) tempered at 45–48°C was added to each plate and mixed with the inoculum. When the agar had solidified, the plates were inverted and incubated for 24 h at 35°C. The original dilutions were stored at 4°C. After incubation, plates with 25–250 colonies were counted, and an estimated count per millilitre of the relevant dilution was established.

Although it was anticipated that limited growth of the culture would occur at 4°C, it was assumed that the figures obtained from the plate counts would be sufficiently accurate to calculate the numbers of colony-forming units (cfu) in inocula prepared from the stored dilutions.

Preparation of the samples: labneh

Bulk samples of freshly-prepared labneh (one with 1·0% salt and one without) were collected from a local dairy on the day of production in sterile containers and delivered to the laboratory in a refrigerated van.

Two batches of salted and two batches of unsalted labneh—each of c. 3·2 kg—were weighed into sterile, stainless-steel buckets (10 l). One batch of salted labneh was then subjected to continuous blending with a slow-speed, electric egg-beater, and the acidity adjusted to pH 4·5 by the stepwise addition of 10% NaOH solution. After each addition of alkali, the labneh was mixed gently for 5 min, and the pH was then recorded with a Kent EIL 7020 Meter and combination electrode; the process was repeated until the desired end-point had been achieved. The second batch of salted labneh was used at its original pH of 3·8. Two similar batches of unsalted labneh were prepared at the same time.

From the original dilution of *L. monocytogenes* that gave an estimate count of $3{\cdot}8\times10^{7}$ cfu ml^{-1}, 1 ml was added to 9 ml peptone water and, after agitation on whirl-mix, the entire culture (10 ml) was added to one of the four batches of labneh; this volume gave an estimated count of 11 800 cfu g^{-1} labneh. The container of labneh was then placed in a laminar-flow cabinet, and the inoculum carefully mixed throughout the product with the electric egg beater. The same procedure was repeated with the three remaining batches to give a final pattern of inoculated products (3·2 kg) of: unsalted pH 3·8, unsalted pH 4·5, salted pH 3·8 and salted pH 4·5.

Because the removal of samples from the bulk material could have lead to 'puddles' of whey forming in the labneh, sub-samples (150–200 g) of each batch were distributed into 18 sterile, screw-cap containers. This allocation allowed for pairs of containers to

be examined for *L. monocytogenes* for 3 successive days after inoculation, and then on alternate days for a typical shelf life (15 days). All the containers were placed in a refrigerator maintained at 4°C until analysed.

On the following 3 days, the entire procedure from collecting the labneh through to setting-up the duplicate sub-samples was repeated, but with the sets of 18 containers being stored at 10, 20 and 30°C.

Enumeration of L. monocytogenes

On each occasion, two screw-cap containers (150–200 g) were selected at random from each temperature regimen, and duplicate samples (25 g) of labneh were weighed from each container into sterile Stomacher bags. Sterile peptone water (225 ml, 0·1%) was added to prepare the first dilution (10^{-1}), and the second dilution (10^{-2}) was prepared by adding 1 ml of the first dilution to 99 ml sterile diluent. Aliquots (0·1 ml) of each dilution were spread onto each of two plates of listeria selective agar (LSA) (Anon. 1994). Typical colonies of *L. monocytogenes*, i.e. colonies with a sunken centre and black colouration of the medium, were counted after 48 h incubation at 35°C. The number of *L. monocytogenes* per gram of labneh was calculated from the average of the four counts (duplicate plates for two samples).

Preparation of the samples: houmos

It emerged during some initial trials that houmos is an excellent growth medium for bacteria, for a survey of freshly-prepared houmos from restaurants included an examination for total bacterial load (aerobic plate count on plate count agar at 35°C for 48 h); coliform count (violet red bile agar at 35°C for 24 h, followed by confirmation in brilliant green bile broth at 35°C for 24–48 h); presence of faecal coliforms (MacConkey broth at 35°C for 24–48 h, followed by confirmation in brilliant green bile broth at 44·5°C in a water-bath for 24–48 h); *Staphylococcus aureus* (Baird–Parker medium at 35°C for 48 h, followed by confirmation of black colonies with or without an opaque zone by the coagulase test); and *Salmonella* (lactose broth at 35°C for 18–24 h followed by selenite broth at 35°C for 24 h; loopfuls of the latter broth were streaked onto Hektoen enteric agar for preliminary identification of genus (Anon. 1994, Richardson 1985).

A specific examination of any naturally-occurring *Listeria* involved weighing 25 g houmos into a sterile Stomacher bag, and adding 225 ml listeria enrichment broth (LEB) (Lovett et al. 1987). The contents of the bag were blended in a Stomacher (Type 400) for 30 s, and incubated at 35°C for 48 h. A loopful was then streaked onto LSA and incubated at 35°C for 48 h (Curtis et al. 1989).

Faecal coliforms were isolated from all 22 samples examined, *S. aureus* from 13 (50–1400 cfu g^{-1}) and *Salmonella* sp. from one sample. The aerobic plate counts (17 000–3·4 $\times 10^6$ cfu g^{-1}) and coliform counts (95–22 000 cfu g^{-1}) were also unacceptable; no *Listeria* spp. were isolated during the survey. The pHs of the samples ranged between 4·7–5·6 and, although no direct correlation emerged, it is likely that the higher values had encouraged microbial development.

It was concluded from this pattern of contamination that: (1) pathogenic bacteria, including perhaps *Listeria* spp., could grow and survive in houmos; and (2) the direct enumeration of *L. monocytogenes* from 'spiked' samples onto LSA could result in contaminants outnumbering the *Listeria*. Consequently, it was decided to use canned houmos as the test material, even though it contained citric acid along with the usual basic ingredients (mashed chick peas and sesame paste). The inclusion of citric acid in the formulation could have rendered the product marginally bacteriocidal *vis-a-vis* fresh houmos, but it was decided that: (1) the risk of low rates of survival of *Listeria* in the canned houmos could be moderated, for experimental purposes, by raising the inoculation rate from 11 000 cfu g^{-1} (labneh) to around 30 000 cfu g^{-1}; and (2) if the organism did survive well in the canned houmos, then it could be assumed that its longevity in freshly-prepared houmos would be extended even more.

One brand was selected on the basis of the ingredient specification and, after wiping the lids of the cans with cotton wool soaked with

70% alcohol and flaming, the cans were opened under sterile conditions; the contents were then transferred to a sterile, stainless steel vessel. The experimental houmos was prepared as directed on the labels of the cans, and this stage involved: (1) washing green chillies, peeled garlic and lemon thoroughly in tap water; (2) grinding these ingredients and a teaspoon of salt into a paste; and (3) mixing the paste into the houmos; a portion was collected for chemical analysis.

Inoculation of the houmos

The preparation of the inoculum was similar to the procedure described for labneh. The APC of the selected dilution was $8{\cdot}28\times10^{7}$ cfu ml^{-1}, so that when 1·0 ml in peptone water (9 ml) was added to 3·0 kg houmos, the anticipated count of *L. monocytogenes* was 27 600 cfu g^{-1} houmos. The inoculated material was blended with the slow-speed egg beater to ensure uniform distribution, and three further batches of houmos was prepared in the same manner. The four batches of 'spiked' houmos were then distributed (150–200 g) into 40 sterile, screw-cap containers. A layer of olive oil (2·5 cm) was poured onto the houmos in 20 of the containers, while the others were left with the surfaces exposed to the air. Five containers from each treatment were then placed in a refrigerator maintained at 4°C, while similar groups were allocated to incubators at 10, 20 and 30°C. This allocation allowed for one container of each treatment to be examined for *L. monocytogenes* at 8, 16, 24, 48 and 72 h after distribution, and the enumeration procedure was the same as that described for labneh.

The number of *L. monocytogenes* per gram of any given sample of houmos was calculated from the average of the four counts (duplicate plates for two samples from each container).

The data were analysed using the General Linear Models procedure of SAS (1987), and differences between treatments were tested using Duncan's multiple range test.

Results and Discussion

After allowing for the slight variations in weight of the labneh, the estimated counts were: 11 800 cfu g^{-1} of salted labneh (pH 3·8 and 4·5); 11 700 cfu g^{-1} of unsalted labneh (pH 3·8) and 11 600 cfu g^{-1} of unsalted labneh (pH 4·5).

After holding the individual containers overnight to reach the required temperatures, analyses of the different samples showed that the numbers of *L. monocytogenes* had been reduced by *c.* 75% (see Table 1). In most cases, this dramatic rate of cell loss continued and, irrespective of the salt content, *L. monocytogenes* did not survive more than 1 day at 4°C or 10°C at pH 3·8. At pH 4·5, some cells survived for 7 days at 4°C and for 5 days at 10°C in unsalted labneh but, in the salted variety, the organism only survived until the fifth day of incubation at 4°C.

Clearly, survival was influenced principally by pH ($P<0{\cdot}05$), with salt ($P<0{\cdot}05$) and temperature applying additional stresses. In particular, one of the reasons for the poor survival of *L. monocytogenes* at the higher temperatures may have been an increasing level of lactic acid generated by the starter bacteria (Tamime 1990). In addition, as it is the undissociated form of lactic acid that is inhibitory to *Listeria* (Hunter and Seigel 1973), the higher temperatures may have encouraged more rapid diffusion into the cells.

It may be relevant also that *L. monocytogenes* is known to be inhibited by the lactic acid bacteria found in starter cultures (Schaak and Marth 1988). In particular, the species of bacterium used in the preparation of labneh, namely *L. delbrueckii* sub-sp. *bulgaricus* and *S. thermophilus* were shown to be inhibitory to *L. monocytogenes* in skim-milk and yoghurt mix. The same workers found that *L. delbrueckii* sub-sp. *bulgaricus* was more inhibitory than *S. thermophilus*, and that the degree of inhibition was affected by both the level of starter addition and the temperature of incubation. It is possible, therefore, that the destruction of *L. monocytogenes* in the present study was the result of the combined action of pH, salt and an inhibitory effect from the starter culture.

Similar studies (Conner et al. 1986, Sorrels et al. 1989) concluded that the inhibition of growth and/or survival of *L. monocytogenes* is a function of acidity and incubation tem-

Table 1. Recovery of *L. monocytogenes* (mean cfu g^{-1}) on *Listeria* selective agar from different samples of labneh inoculated with estimated counts (see text) in excess of 11 000 cfu g^{-1} and held at the temperatures indicated

Temperature		Time from inoculation (days)				
		1	2	3	5	7
4°C	Salted/pH 3·8	1550	125	0	0	0
	Salted/pH 4·5	3000	1875	675	275	0
	Unsalted/pH 3·8	3250	200	0	0	0
	Unsalted/pH 4·5	3150	2800	1325	275	25
10°C	Salted/pH 3·8	1500	150	0	0	0
	Salted/pH 4·5	3000	475	0	0	0
	Unsalted/pH 3·8	3250	275	0	0	0
	Unsalted/pH 4·5	3150	2100	475	250	0
20°C	Salted/pH 3·8	1550	0	0	0	0
	Salted/pH 4·5	3000	25	0	0	0
	Unsalted/pH 3·8	3250	25	0	0	0
	Unsalted/pH 4·5	3150	550	0	0	0
30°C	Salted/pH 3·8	1550	0	0	0	0
	Salted/pH 4·5	3000	0	0	0	0
	Unsalted/pH 3·8	3250	0	0	0	0
	Unsalted/pH 4·5	3150	0	0	0	0

Table 2. Recovery of *L. monocytogenes* (mean cfu g^{-1}) on *Listeria* selective agar from samples of houmos inoculated with estimated counts of 27 000 cfu g^{-1} and held at the temperatures indicated; 50% of the samples were covered with olive oil immediately after inoculation

Temperature		Time (h)					
		0	8	16	24	48	72
4°C	With oil	27 000^{a}	23 000^{bc}	24 000^{b}	19 400^{d}	21 500^{c}	19 100^{d}
	Without oil	27 000^{c}	29 000^{b}	22 000^{d}	27 000^{c}	27 500^{bc}	32 500^{a}
10°C	With oil	27 000^{a}	23 500^{b}	24 000^{b}	13 900^{c}	12 100^{d}	12 100^{d}
	Without oil	27 000^{ab}	25 500^{b}	25 000^{b}	26 000^{b}	28 000^{ab}	30 000^{a}
20°C	With oil	27 000^{a}	26 000^{a}	22 500^{b}	15 500^{c}	10 000^{d}	9000^{d}
	Without oil	27 000^{a}	27 500^{a}	28 000^{a}	22 000^{b}	8000^{c}	9000^{c}
30°C	With oil	27 000^{b}	30 000^{a}	21 500^{c}	9850^{d}	7000^{e}	8000de
	Without oil	27 000^{b}	30 000^{a}	21 000^{c}	12 000^{d}	8500^{e}	8000^{e}

[a]Means within a row lacking a common superscript differ, significance $P<0·05$.

perature, and Parish and Higgins (1989) showed that there was a decline in numbers of *L. monocytogenes* at pH<4·5. Consequently, it is not surprising that *Listeria* cannot survive long in labneh, or that the product has a good record with respect to consumer safety. Nevertheless, more than 1000 cfu g^{-1} did survive for 3 days in unsalted labneh at pH 4·5, and hence gross contamination in the home could pose a marginal risk to susceptible individuals.

The results of the analyses of the houmos

are shown in Table 2, and it is noticeable that, in houmos stored without olive oil, the cell counts showed a slight increase in number at 4°C over the 72 h period of incubation. At 10°C, the counts remained stable, whereas at 20°C and 30°C, the counts had declined significantly ($P<0.05$) after 16 h. Once again, survival of the cells was best at low temperature, so that chilled fresh houmos contaminated with *Listeria* would remain a risk within its expected shelf life of 24 h. At higher temperatures, it could be that the citric acid in houmos is exercising a bacteriocidal effect but, unless the product is subject to severe temperature abuse, the inhibition will be of little benefit to the retailer and consumer.

When the houmos was covered with oil, the counts decreased significantly ($P<0.05$) over 72 h at all temperatures, although the decline was sharpest at ambient temperatures. The significant difference ($P<0.05$) between the overall counts with or without olive oil may have been a result of an antimicrobial effect of olive oil *per se* (Raina 1993) but, although *Listeria* spp. are facultative anaerobes, the exclusion of oxygen could have affected survival of the strain in question.

Overall, it was evident that, although an acidic product like labneh can be regarded safe, with respect to *Listeria*, high counts of *L. monocytogenes* are able to survive in houmos for up to 3 days—the maximum expected shelf life of the product. Obviously the initial counts employed in this trial were much higher than should be reached by casual handling in a kitchen. Nevertheless, the analysis of normal retail products suggested that houmos is prone to contamination during preparation, so that steps to avoid any contact with *Listeria* should be taken seriously by producers.

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Comparison of techniques for measuring the rheological properties of labneh (concentrated yogurt)

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The effectiveness of conventional rheological techniques (destructive) and the oscillatory dynamic test (non-destructive) for the study of the physical properties of concentrated yogurt (labneh) was studied. Six different types of labneh (control (cloth bag method), ultrafiltrated (UF)-after and -before fermentation, reverse osmosis (RO)-after and -before fermentation and direct reconstitution from whole milk powder) were examined. Dynamic rheological studies revealed that labneh is a viscoelastic system in which its elastic characteristic is more dominant than its viscous properties. The elastic and viscous attributes of the control labneh were significantly different from the rest of the test samples. In general, the samples with low protein content (RO-after and -before fermentation and direct reconstitution labneh) produced weaker gel structures than their UF counterparts. The penetrometer and viscometer (destructive techniques) failed to reveal expected differences between the samples, and the results did not correlate with the oscillatory dynamic tests. In the light of these results, it could be suggested that dynamic studies are much more reliable than the destructive rheological techniques for the study of the physical properties of labneh.

INTRODUCTION

The yogurt gel is a heat induced acid casein gel, and consists of a permanent network composed mainly of non-covalent protein bonds (eg, hydrophobic and electrostatic binding) as well as covalent thiol-disulphide bonds. There are many factors which affect the physical properties of yogurt, such as the type and number of the protein interactions, the size and shape of the protein network and the distribution of whey proteins and caseins in the aqueous phase. All these factors are affected by the technical applications, such as heat treatment, incubation temperature and pH, type of starter culture, methods of manufacture, as well as type of milk.[1]

Because this combination of factors plays a determinative role in the rheology of the resulting gel, the method which is selected to study the physical properties of yogurt should, ideally, be sensitive enough to measure the viscoelastic properties of the test samples without disturbing the nature of the gel forming components.

Over the last two decades, conventional techniques, such as the Plummet device,[2] the Posthumus funnel,[3] the falling ball,[4] the Namatre vibrator[5] and the Rheomat,[6] have been almost universally accepted for the measurement of the physical properties of set or stirred yogurts, and rotational viscometers, such as the Haake[7] and the Brookfield[8] have become widely used as well.

For set yogurt, different types of penetrometer/consistometer, such as the curd tension-meter,[9] Instron testing machine,[10] Stevens Texture Analyser[11] and the SUR-penetrometer PNR[12] have been widely used to assess the firmness of the body/gel.

However, traditional techniques (1) measure the physical properties of residues of the gel after mechanical agitation, (2) give single point measurements (fixed rate, strain or both) which do not mirror the actual rheological characteristics of yogurt and (3) cannot provide sufficient data to calculate the exact shear rate/strain for stirred yogurt.[13]

Consequently, a great deal of research has investigated the rheology of normal, set- and stirred-yogurts with the non-destructive dynamic rheometer.[1,14–20] In particular, the weak viscoelastic nature of yogurt gel is well established[1,14] and the rheological properties of yogurt can be explained by measuring its viscous and elastic moduli. The most widely employed viscoelastic tests are creep, stress relaxation and dynamic oscillatory testing.[15] However, while the stress relaxation and the creep tests are mostly used to measure the rheological characteristics of stationary gel networks, such as cheese, the dynamic oscillatory tests are more suitable for monitoring the gel properties of time dependent gel systems. Nevertheless, the gel properties of concentrated yogurt have not been subjected to dynamic oscillatory tests. Thus, the aim of the

Original paper.
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present work was to study the effect of increasing total solids by various techniques on the rheology of concentrated yogurt. Additionally, the comparability of traditional techniques and the dynamic oscillatory test for the study of the rheology of concentrated yogurt was investigated.

MATERIALS AND METHODS

Materials

Full fat milk powder supplied from Adams Food Ingredient Ltd (Staffordshire, UK) was used in the production of the test samples. The powder was stored at 4°C until used.

A freeze dried yogurt culture (coded CH-1) from Chr Hansen's Laboratory (Reading, UK) was used in the manufacture of yogurt. The starter was a blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* in equal portions.

Methods

Six different labnehs were analysed. Labneh made by the traditional cloth bag method, ie, hanging yogurt having 16% total solids in a double layer cheese cloth bag for about 18–20 hours at 4°C until the final product reached 23%, was chosen as the control.

Ultrafiltration was employed either before or after fermentation, and the products are referred to as UF-before fermentation labneh and UF-after fermentation labneh, respectively. The UF cartridge consisted of a bundle of tubular membranes, surface area 0.8 m^2; type ES 625, Patterson Candy, Whitchurch, Hants, UK, polyether sulphone; nominal molecular weight cut-off 25 000 daltons.

Reverse osmosis was applied either before or after incubation, and the products are referred to as RO-before fermentation labneh and RO-after fermentation labneh, respectively. The specifications of the RO membrane were: surface area 1.2 m^2; type ZF 99, Patterson Candy, membrane composed of polyether sulphone.

During the application of membrane processes (both UF and RO) the temperatures of milk and yogurt were maintained at 50°C and 45°C, respectively. The inlet and outlet pressures for the UF plant were 4 and 2 bar, respectively; the RO plant was operated at 20 bar for yogurt and 25 bar for milk systems.

The last treatment was produced by reconstituting the required amount of full cream milk powder in softened water at 40°C (direct reconstitution labneh).

The yogurt making procedure proposed by Tamime and Robinson[2] was followed. The initial total solids level of the milks and yogurts was 16%, and after concentration 23% total solids; the standard heat treatment was at 85°C for 20 minutes. Acidification was achieved by inoculation with the starter culture (2% v/v) at 43°C. Incubation was halted when the pH dropped to 4.3 for samples concentrated postincubation, and 4.0 for previously concentrated samples; the final pH of all samples was 4.0.

Determination of the gel firmness was by means of a Stevens Texture Analyser (C Stevens and Son Ltd, Herefordshire, UK), fitted with a chart recorder model BS 271. A cylindrical probe (1.2 cm in diameter and 4.5 cm in height) was used. The speed of the probe, penetration depth and chart speed were 0.5 mm s^{-1}, 15 mm and 30 mm min^{-1} at 200 mV, respectively.

The apparent viscosity was measured with a Brookfield rotational viscometer (Brookfield Engineering Laboratories, Inc, Massachusets, USA), model LVT, with a helipath stand. Approximately 350 ml of yogurt sample was analysed at low rotation speed, ie, 0.6 rpm with T-bar spindles.

The dynamic rheological studies were carried out using a stress-controlled rheometer (Rheotech International, UK). The rheometer was set up with a parallel-plate (10 mm radius and 1 mm gap setting). The temperature of the samples was maintained at 25°C using a circulating water system.

Statistical evaluations were completed using the Excel software program, and statistically significant groups were determined by the Duncan test.

TABLE 1

Chemical composition of the test samples; all figures as g/100 g of sample

Samples	*Total solids*	*Protein*	*Lactose*	*Fat*	*Total mineral*
Control	23.31	8.0	5.16	9.18	0.79
UF-before	22.44	8.25	5.21	8.20	0.78
UF-after	22.64	8.13	5.50	8.45	0.86
RO-before	23.22	6.82	8.98	6.25	1.08
RO-after	22.22	6.38	8.84	6.60	1.00
Direct reconstitution	22.50	6.38	8.72	6.10	1.30

RESULTS AND DISCUSSION

Preliminary studies indicated that labneh (concentrated yogurt) is a typical weak viscoelastic gel system in which the elastic modulus (G') is greater than the loss modulus (G").

Variations in the viscoelastic components of the test samples over the range of amplitudes applied are presented in Figs. 1 and 2. Strikingly, none of the treatments produced the same gel properties as the control sample, in spite of the fact that UF-before and -after fermentation labnehs had similar chemical composition to the control (see Table 1). This significant difference between the control and the rest of the test samples may result from the compact structure of the control sample resulting from gravity drainage. UF-before and -after fermentation labnehs to some

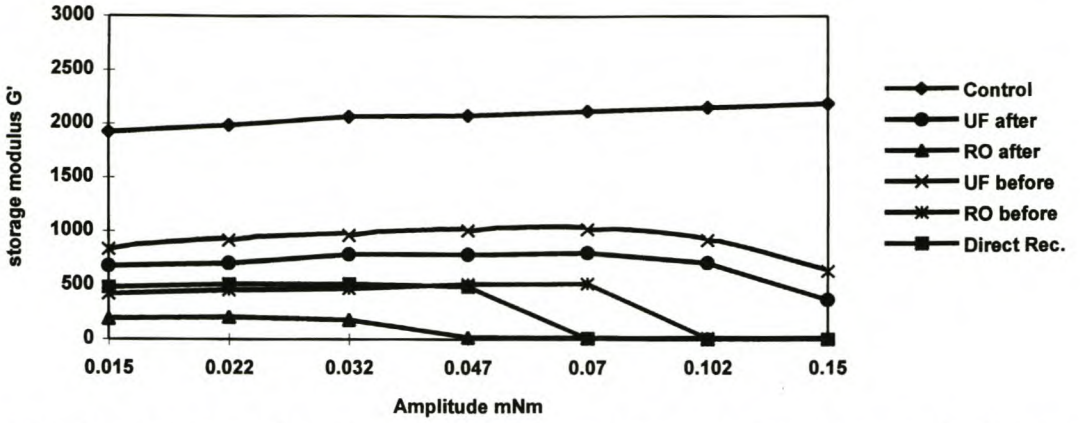

Fig. 1. Typical storage modulus pattern of labnehs after overnight storage. Results are the average of eight separate runs (standard errors less than symbol dimensions). Test conditions are: amplitude range 0.015–0.15 mNm, frequency 0.25 Hz, parallel-plate (10 mm radius and 1 mm gap setting), 25°C measuring temperature. Direct Rec. = direct reconstitution.

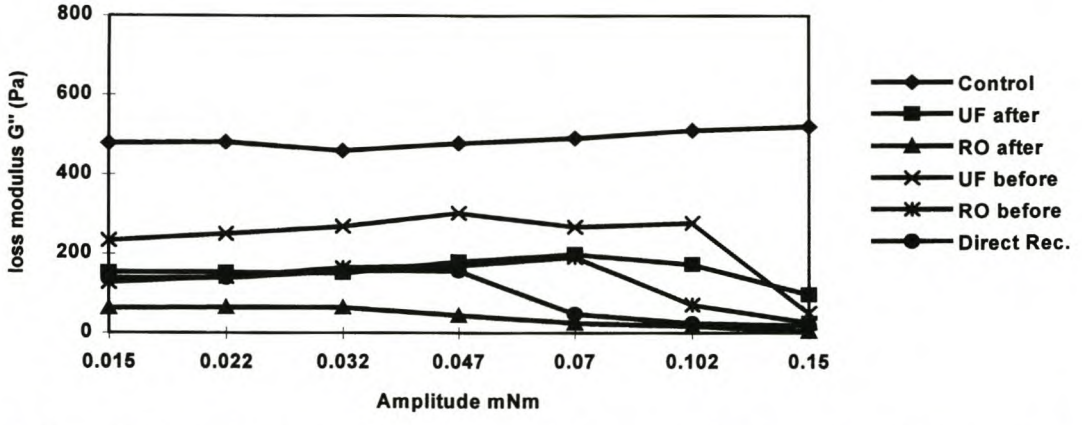

Fig. 2. Typical loss modulus pattern of labnehs after overnight storage. Results are the average of eight separate runs (standard errors less than symbol dimensions). Test conditions are: amplitude range 0.015–0.15 mNm, frequency 0.25 Hz, parallel-plate (10 mm radius and 1 mm gap setting), 25°C measuring temperature.

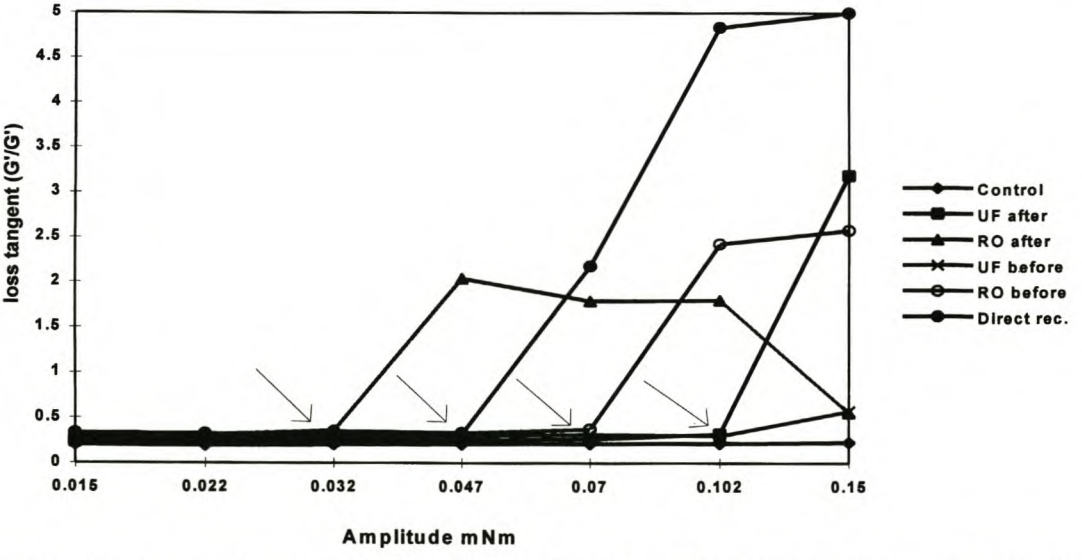

Fig. 3. Variation in loss tangent values of the samples as a function of amplitude. Arrows indicate the breaking point of structure of each sample.

extent kept their structural integrities against increasing shear. However, the rest of the samples broke down at some point within the range of amplitude applied. After the gel structure broke down, the viscous character of the samples became dominant. This effect is better seen in Fig. 3, which shows the variation in loss tangent values ($\tan \delta = G''/G'$) as

a function of amplitude. Roefs[21] suggested that the tan δ value is related to the nature of the bonds forming the protein network and the relative importance of the different types of bond rather than to the spatial distribution of protein junction points. As the type of protein and the gelation conditions were identical in each system, it is reasonable to deduce that the different treatments for increasing total solids led to the differences in the levels of protein and hence relative dominance of the bonds forming the protein network.

In terms of the elastic and loss moduli, UF-before and -after fermentation labnehs were similar. The direct reconstitution and RO-before fermentation labnehs had very similar gel properties, but were weaker than the control and UF samples. The destructive effect of excess mechanical force (20 bar pressure) on the gel structure of the RO-after fermentation labneh was remarkable. This sample had a very weak and sponge-like structure, giving it an atypical appearance for labneh.

The firmness of the samples examined by the Stevens Texture Analyser is illustrated in Fig. 4. The results indicated that there was a clear difference between the control and the rest of the test samples, but the penetrometer failed to reveal possible differences between the other samples. Thus the elastic moduli of UF treated samples (Fig. 1), for example, were some 4–5 times higher than that of RO-after fermentation labneh, but the gel strengths of these samples were not significantly different ($p > .05$).

The control and the UF-before fermentation labnehs had more viscous characteristics than the others (Fig. 5), but little correlation between the penetrometer and viscometer measurements was found. This lack of correlation corroborates the theory that destructive techniques cannot mirror the actual rheology of yogurt, which has a viscoelastic nature. In other words, each penetration into or rotation in a gel network causes a breakdown in the elastically effective bonds, and the procedure thus fails to measure the actual physical characteristics of the gel. Additionally, as compared to literature data on model casein gels and microstructural findings on yogurt,[11] the results imply that

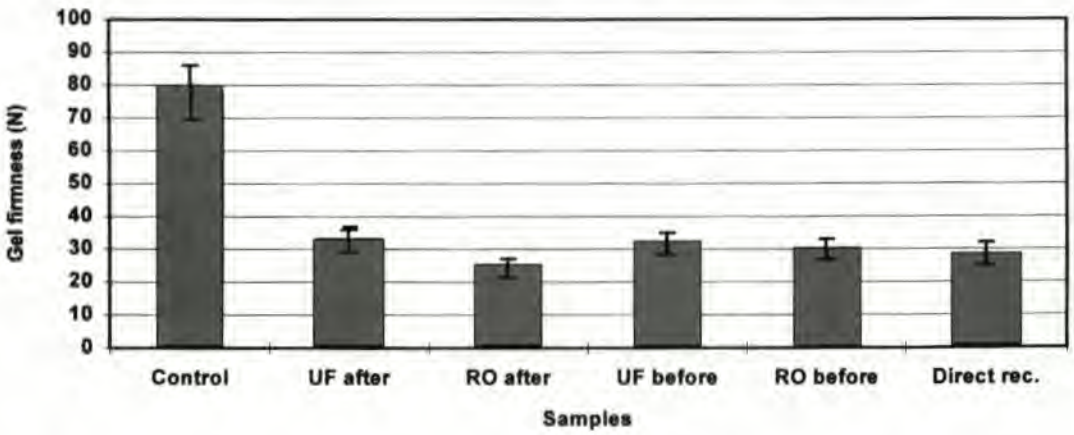

Fig. 4. Gel firmness of test samples measured by Stevens Texture Analyser. Results are the average of three separate runs.

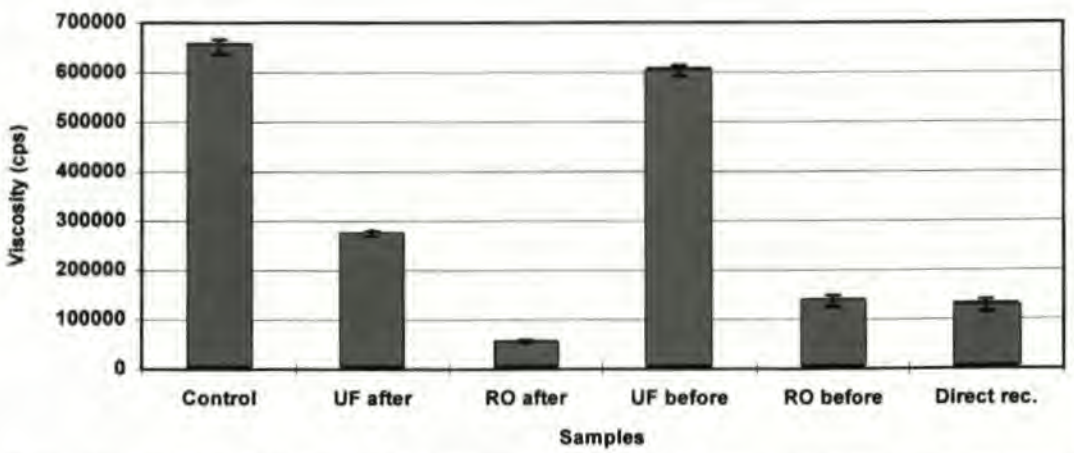

Fig. 5. Viscosity values of the test samples after overnight storage at 4°C. Results are the average of three separate runs.

yogurt is a heterogeneous particulate gel. Therefore, single point measurements are insufficient to represent the rheology of the entire gel.

CONCLUSIONS

Dynamic rheological tests (oscillatory tests) are more suitable for the study of the rheology of concentrated yogurt than traditional techniques. The key point is to preserve the natural form of the gel as long as possible. Once the gel structure is disturbed, it is rarely possible to re-form the gel structure in the same way again, because yogurt is a metastable gel and any change in its enthalpic/entropic nature creates irreversible deformation. Thus, any kind of destructive effect may lead to atypical physical properties in the yogurt, and provide erroneous results.

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Consumer interest in yogurts with health promoting properties is slow to arise in the UK. R K Robinson examines the therapeutic and/or prophylactic values of these 'special' cultured products

Special yogurts
the potential health benefits

According to most reports, the market for yogurt in the UK is buoyant, indeed still expanding and it is of note that at least part of this expansion has centred around the introduction of new lines.

The growth in demand for 'Greek style', natural yogurts or the 'rich and creamy' fruit yogurts provides examples of this trend and yet British consumer interest in one group of new products remains steadfastly dormant.

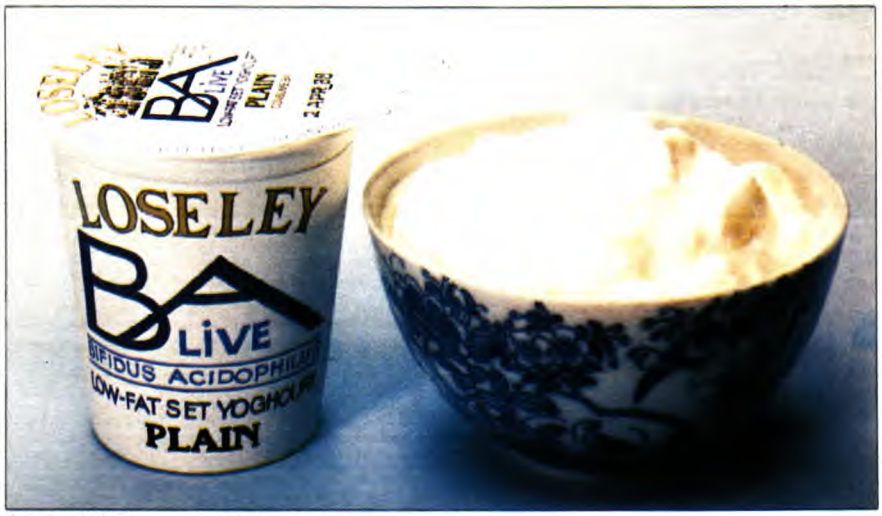
This latter group consists of yogurts that contain cultures with alleged health-promoting properties, or new lines that are fermented with similar cultures, but ones that do not include *Lactobacillus delbrueckii* sub-sp *bulgaricus (L. bulgaricus)*; thus, the retail items are not 'yogurts' *sensu strictu* (1).

In Germany, recently introduced regulations permit these latter products to be referred to as 'mild yogurts' (2), for while they lack the precise microflora associated with the traditional material, they are in most other respects, indistinguishable from a 'low-acid' yogurt. However, whatever the preferred designation, selling the concept of 'health benefits' can, in the UK, be a curiously daunting task (3).

Elsewhere in the world, the situation is markedly different. In France, for example, 'mild yogurts' account for around 10% of the total yogurt market (4), while in Germany, yogurts containing therapeutically desirable species of bacteria are readily available.

In the Far East, sales of the fermented milk, Yakult, testify to the growing interest in 'healthy eating' and the same concern is shown in the United States, where products containing *Lactobacillus acidophilus* have long enjoyed a niche in the market place.

In respect to the UK, however, sales of these 'special' yogurts are of marginal importance, but for reasons that are not altogether clear. Certainly neither the resistance of some multiple stores to 'health-promoting' lines nor, on occasions, a substantial price differential in favour of standard yogurts have helped. The failure of manufacturers to provide a full-range of fruit flavours, even though there is no evidence that natural flavours, such as strawberry, would affect the viability of the special starter cultures (5), may also have proved a disincentive for some potential buyers. Finally, of course, there is a lack of conviction in certain quarters that the added bacteria have any therapeutic or prophylactic value (6).



Loseley BA Live was introduced last year.

Comment on the marketing of these products is beyond the intended scope of this paper and hence discussion will be limited to attempting to counter the last criticism. In particular, attention is paid to summarising the potential benefits that may accrue from the regular consump-tion of these 'special' cultured products.

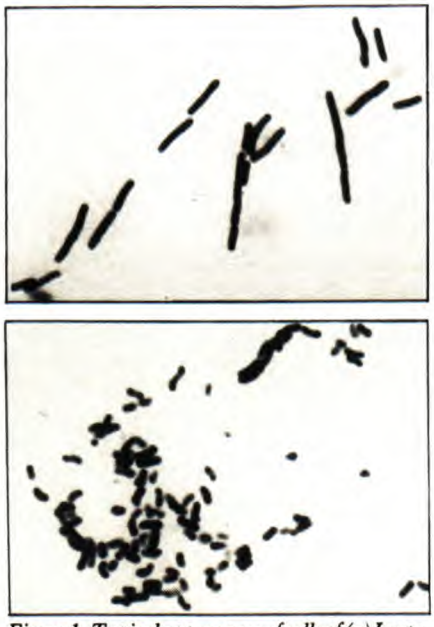


Figure 1. Typical appearance of cells of (a) Lactobacillus acidophilus and (b) Bifidobacterium bifidum taken from cultures growing in skim-milk. (Courtesy of Mrs Debbie Wapshott and Mr C. W. Varnals).

The important species

Although *Lactobacillus casei* is employed for its therapeutic properties in the production of Yakult, the major interest in Europe centres on *Lactobacillus acidophilus* and two species of *Bifidobacterium,* namely *bifidum* and *longum.* Strains of these three species occur as natural components of the intestinal microflora of humans and it is their possible activity within this habitat that is of especial interest.

Both genera are capable of producing lactic acid from lactose and/or other sugars and they appear under the microscope as Gram-positive rods (figure 1). The morphology of the cells does alter with habitat/medium, but the examples shown are fairly typical of their appearance in cultured milks.

L. acidophilus and the two species of *Bifidobacterium* are frequently grown alone in milk (see table 1), but they can also be present along with a normal yogurt culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The main advantage of this approach is that the fermentation process is much faster than when the special species are employed alone, but there is also evidence that the yogurt culture encourages the desired activity of *L. acidophilus* and the bifidobacteria within the intestine (7).

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Table 1. Some examples of yogurts and 'mild yogurts' (see text) that contain cultures with alleged health-promoting properties (2, 4, 16)

Brand name	Species of bacterium present			
	Latobacillus acidophilus	bulgaricus	Bifidobacterium bifidum/longum	Streptococcus thermophilus
A-B Yogurt	+	+	+	+
B-Active	+	—	+	—
BA-live	+	—	+	—
BA-nature	+	+	+	+
Biogarde	+	—	+	—
Bifighurt	—	—	+	—
Bioghurt	+	—	—	+
Cultura	+	—	+	—
Real Active	—	+	+	+

Employing this latter approach, it is important, however, that both *L. acidophilus* and the species of *Bifidobacterium* are susceptible to prolonged exposure to low pH. For this reason, either the pH of the end-product has to be controlled to remain above 4.6-4.7, or the initial numbers of the special species have to be sufficiently high that, even after storage at pH 4.1-4.2 for two/three weeks, the product will still contain a minimum of 10 x 10^5 viable cells/ml (8). Counts of this order are usually referred to as the 'therapeutic minimum' and it is essential that products sold under the 'health promoting' banner meet this criterion (9).

Obviously, the brands mentioned in table 1 have achieved considerable popularity as natural, 'mild yogurts', but for fruit or fruit flavoured variants, it may be that the optimum approach will be to produce a yogurt that contains either *L. acidophilus* or *Bifidobacterium* spp in addition to the normal starter. If such products were to make an appearance on the supermarket shelves in the UK and at a price equivalent to normal yogurt, then the question arises — could a consumer expect to derive a health benefit from the regular consumption of this type of product?

Some likely properties of the special yogurts

It must be admitted at the outset that the properties attributed to these special yogurts have been established only on the basis of limited clinical trials and some physicians believe that a normal, healthy adult will derive little benefit from the intake of the 'live' cultures involved.

Nevertheless, so many papers have now been published citing evidence of the 'health-promoting' properties of *L. acidophilus* and *Bifidobacterium* spp that it is difficult to dismiss the findings as chance observations. In many cases, of course, it is fair to point out that the test subjects are, in a sense, 'abnormal' eg as patients suffering from the after-effects of antibiotic therapy or from viral or other infections, but the benefits that they have derived from the ingestion of these special products are, nonetheless, real enough.

Some of the observed and/or potential benefits are summarised below:

— Assuming that the base milk has been fortified prior to manufacture, then the consumer will be receiving an enhanced protein intake *vis-a-vis* normal milk and in a form that is more rapidly digested (10, 11). It is also possible that some of the mineral components, eg calcium, may be more readily available.

— Although the lactose content of these products is often similar to, or indeed above, that of liquid milk, there is clear evidence that 'lactose maldigestors' can eat yogurt with little adverse affect (12).

— It is widely accepted that the consumption of normal yogurt on a routine basis can lead to a reduction in the level of serum cholesterol in certain individuals (10). The exact reasons for this reduction are not clear, but it seems likely that the inclusion of *B. bifidum* or *L. acidophilus* in a product would enhance this activity.

— A number of genera of food-borne bacteria, such as *Salmonella* and *Campylobacter* contain species which can cause severe and distressing symptoms as a result of activity within the gastro-intestinal tract. The routine intake of yogurt containing the special cultures could well eliminate the consequences of ingesting these undesirable bacteria, or at least minimise their impact (13, 14). Some reasons for proposing this effect are:

(a) a number of lactic acid bacteria, including *S. thermophilus* and *L. bulgaricus*, are reported to produce low levels of antimicrobial compounds that are effective against pathogens like *Salmonella;*

(b) these same species stimulate the growth of bifidobacteria in the intestine and hence their ingestion will help to ensure that species of *Bifidobacterium*, whether naturally resident or ingested concomitantly with the yogurt organisms, will dominate the microflora of the gut;

(c) the presence of large numbers of resident bifidobacteria and, perhaps, lactobacilli will provide active competition against invading pathogens for both space, eg sites for attachment to the intestinal wall, and nutrients. In addition, the sphere of influence of the desirable, resident bacteria will include the secretion of lactic acid, and in some cases, other metabolites that will inhibit the activity of the pathogenic organisms.

These same activities will also help prevent the 'overgrowth' of the intestinal flora by yeasts or *E. coli* consequent upon illness or certain types of therapy (15).

— One of the long-term effects of maintaining bifidobacteria as the dominant organisms in the large intestine is the prevention of colonisation by the so-called putrefactive bacteria. These latter organisms are capable of breaking-down food residues to give waste products that are toxic to the human body; any mechanism that restricts or nullifies this activity therefore could prove beneficial. The degradation of nitrosamines by the bifidobacteria is a case in point, for these compounds are amongst those reported to be carcinogenic (13, 15).

It is reported also that the regular consumption of 'live' yogurt can stimulate the immune system of humans and the presence of *L. acidophilus* or *Bifidobacterium* spp might well encourage this development (14). Obviously, the response of any given population to the consumption of one of the special yogurts cannot be predicted accurately.

Nevertheless, more and more incidents of 'improved health' following consumption of these products are being reported and it is hard to justify the current scepticism about their value as health-promoting, dietary adjuncts. Whether consumers in the UK will begin to follow their counterparts in continental Europe and demand the option of selecting yogurts or 'mild yogurts' with special microfloras remains to be seen, but there seems to be little doubt that it is a trend that should be encouraged. □

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A preliminary appraisal of the effect on the cholesterol content of fresh ovine milk of supplementing the feed of Awassi ewes with *Lactobacillus acidophilus*

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1. Introduction

Milk has held a valued place in the human diet for thousands of years, and for the reason that it contains more than 85 g/kg solids-not-fat including >30 g/kg protein, 45 g/kg lactose and a variety of nutritionally important mineral salts. Around 35 g/kg fat is also present and, although the cholesterol content of milk is lower than the level in other animal foods, the figures for some manufactured products like cheese and butter are relatively high (1). Some investigators believe that an excessive concentration of cholesterol in the diet should be regarded as a high risk factor with respect to coronary heart disease (2), and the emphasis today remains in favour of low fat/low cholesterol foods (3), or on the introduction of dietary changes that may lower serum cholesterol. In humans, this latter approach usually involves the ingestion of selected fermented milks, for a number of lactic acid bacteria of intestinal origin have the ability to assimilate cholesterol (4). Whether or not *Lactobacillus* spp. have the same impact on cholesterol in the intestines of other animals is not clear, but HADDADIN *et al.* (5) and ABDULRAHIM *et al.* (6) showed that stimulating the populations of lactobacilli in the intestines of chickens lead to a drop in the levels of cholesterol in both the blood and the eggs of the test birds. Little work appears to have been done on the feeding of probiotic cultures to ruminants (7).

Nevertheless, milk production is a major facet of farming and, given the interest in dietary levels of cholesterol and the criticism of milk and milk products as potential sources, this project sought to follow the impact of feeding *L. acidophilus* to ewes over a period of 120 d with respect to: (i) yield of milk; (ii) serum cholesterol levels; and (iii) concentration of cholesterol in the milk.

2. Materials and methods

2.1 Handling of the cultures

As no cultures of *L. acidophilus* from sheep were available, 4 isolates were retrieved from the crop, the duodenum, the small intestine and the caecum of wild chickens, and an additional culture was obtained from the faeces of a human infant; the 5 isolates were maintained by weekly transfers in MRS Broth and, after growth at 37 °C for 24 h, the cultures were held in the refrigerator at 4 °C. The cultures were tested for identity according to the morphological, physiological and biochemical tests cited in Bergy's Manual of Determinative Bacteriology (8). Once confirmed as strains of *L. acidophilus*, each isolate was screened using the protocol of HADDADIN *et al.* (9) for: (i) tolerance of bile salts (up to 3 g/l of Oxoid Bile Salts No. 3 in MRS Broth (Unipath Ltd., Basingstoke, RG24 8PW, UK); (ii) assimilation of cholesterol from MRS Broth with sheep serum added to give 0.4–0.6 g/l of cholesterol; and (iii) an adhesion test (10) which involved soaking (20 min at 37 °C) slices (100 g) from the small intestine of a sheep in MRS Broth containing a suspension of *L. acidophilus*, washing the slices 3 times in sterile water, and then recording the total colony count of *L. acidophilus*/g of homogenised tissue for comparison with the count in the third wash water.

After screening, the ability of the individual cultures to grow in autoclaved (110 °C for 10 min) non-fat milk (170 g/l of skim-milk powder) and withstand freeze-drying for use in the field was tested. Duplicate batches of each culture were grown in skim-milk over a period of 16 h at 37 °C and then freeze-dried (see below); total colony counts/ml of each culture were recorded every 2 h during incubation, and then/g of the dried powders. On each occasion, duplicate sub-samples (0.1 ml) from

dilutions 10^{-3}–10^{-7} were spread onto the dried surfaces of pre-poured plates of MRS Agar, and the plates were incubated at 37 °C in anaerobic jars with the atmospheres maintained with gas packs from Oxoid (Unipath Ltd., Basingstoke, RG24 8PW, UK).

2.2 Culture for the feeding trial

Individual batches of reconstituted milk (500 ml) were inoculated at a rate of 50 ml/l from 'feeder' cultures of each isolate grown in skim-milk (170 g/l of skim-milk powder) and, after incubation at 37 °C under a constant stream of nitrogen for 16 h, the fermented milks were dispensed into sterile, round bottomed, freeze-drier flasks (250 ml) under aseptic conditions; each flask was placed in a deep-freeze at –30 °C prior to freeze drying. Next day, the flasks were connected to the manifold of a freeze drier (Edwards High Vacuum, Crawley, West Sussex RH10 2LW, UK), and left with a vacuum of 0.007 MPa for 24 h. Each separate batch of freeze dried powder was then ground using a sterile blender (Moulenix, Paris, France), and the total volume blended to give one composite powder containing all 5 isolates of *L. acidophilus*. This final blend was then packed manually, under a Laminar Flow Cabinet (MDH Ltd., Andover, SP10 5AA, UK), into Size 0 (500 mg) enteric capsules (Cornelius, Bishop's Stortford, CM23 5RG UK); these capsules were selected as the coating is resistant to acidic media, but dissolves under alkaline conditions as might be found in the lower intestine of sheep. The filled capsules were kept in the freezer at –18 °C, and duplicate capsules were tested, as described above, every 2 weeks to ensure that the total viable count of *L. acidophilus* did not decline.

2.3 Field trial

The capsules required for feeding to the ewes were removed from the deep-freeze and distributed into Petri dishes to be hardened by spraying with formaldehyde solution (50 g/l) from a fine-droplet manual sprayer. After 5 min, the capsules were collected and spread on a clean plate for air-drying overnight at ambient temperature; on each occasion, 2 capsules were selected at random to check that the treatment with formaldehyde did not affect the total viable count of *L. acidophilus*.

The Awassi sheep and their lambs were housed at the Agricultural Research Station in the Jordan Valley between December, 1997 and April, 1998, and 30 Awassi ewes were randomly divided, following lambing, into 2 groups. Each group of 15 ewes was housed for a period of 16 weeks in a separate corral, and the suckling lambs were with their respective mothers for 8 weeks. The ewes in each group were offered a concentrate of barley, wheat bran, soybean meal and a vitamin and mineral mix at a level of 1.25 kg/d, along with 1.25 kg of roughage consisting of 50% wheat straw and 50% alfalfa hay/d. Every ewe in the experimental group was given, through the oesophagus, a daily dose of 2 capsules containing a total of 1.0×10^9 colony-forming units of *L. acidophilus*. This supplementation was carried out for 16 weeks after lambing, while the remaining ewes were kept as a control group.

2.4 Analysis of progress

Samples of milk were collected weekly from all the ewes for the first month of the experiment for cholesterol analysis, and then every 2 weeks for the following 3 months; at the end of weaning, all the ewes were milked daily to record the total yields/ewe/week over a period of 8 weeks. Blood samples from the jugular vein of each ewe were collected weekly in the first month of the experiment, and then every 2 weeks using heparinized vacutainers (Becton Dickinson Vacutainer System, Grenoble, France). The samples were centrifuged at 5000 rpm for 10 min to collect the serum. Measurements of cholesterol in the blood samples were determined using a colorimetric/enzymatic method (Diasystems International, Hanover, Germany), while the cholesterol content in the milk samples was determined according to the procedure cited in the Boehringer Mannheim Guide for Food Analysis (11).

2.5 Statistical analysis

The results were analysed using the General Linear Model of Statistical Analysis Systems (12), and the means were compared using the Least Significant Difference Method (LSD).

3. Results

All 5 isolates were found to be long, Gram-positive, non-motile, non-sporulating, catalase-negative rods, and pure cultures of each isolate were then subjected to a range of physiological and biochemical tests (8) (Table 1). These tests confirmed that the isolates were strains of *L. acidophilus* and, although they were all of animal origin, the cultures were examined for specific characteristics that would suggest their suitability for a feeding trial.

Table 1: The physiological and biochemical reactions of the 5 isolates of *L. acidophilus* employed in the feeding trial

Test	Reactions of test cultures 1	2	3	4	5	Expected[a]
Growth at 15°C	–	–	–	–	–	–
Growth at 45°C	+	+	+	+	+	+
NH_3 from arginine	–	–	–	–	–	–
Utilisation of						
Aesculin	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Glucose (acid)	+	+	+	+	+	+
Glucose (gas)	–	–	–	–	–	–
Lactose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Mannitol	–	–	–	–	–	–
Mannose	+	+	+	+	+	+
Melizitose	–	–	–	–	–	–
Melibiose	+	–	–	+	+	+/–
Raffinose	–	–	+	–	+	+/–
Rhamnose	–	–	–	–	–	–
Ribose	–	–	–	–	–	–
Salicin	+	+	+	+	+	+
Sorbitol	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+
Xylose	–	–	–	–	–	–

[a] Data from Rogosa (1974); + positive; – negative; +/– variable reaction. Origin of isolates: 1: crop of chicken; 2 duodenum of chicken; 3: small intestine of chicken; 4: caecum of chicken; 5: human infant faeces

Table 2: Some specific attributes of the 5 isolates of *L. acidophilus* used in the present trial

Test	Reactions of test cultures 1	2	3	4	5	Control
Tolerance of bile salts (cfux10^7/ml of *L. acidophilus* after 24 h growth at 37°C in MRS broth + 3 g/l bile salts	15.0^b	10.4^c	5.4^d	4.2^d	19.4^a	53.3
Cholesterol assimilation (% reduction over 24 h following growth at 37°C in MRS broth + sheep serum	65^d	85.1^b	70.8^c	81.6^a	68.2^d	–
Adhesion (cfux10^5/g of *L. acidophilus* in intestinal macerate – control equals count in washing water)	2.5^a	1.2^d	1.5^c	1.0^d	2.2^b	0.5
Growth in milk (cfux10^8/ml of *L. acidophilus* after 16 h growth at 37°C in skim milk)	2.0	1.64	1.32	1.47	1.70	–

Origin of isolates: 1: crop of chicken; 2: duodenum of chicken; 3: small intestine of chicken; 4: caecum of chicken; 5: human infant faeces
Note: Means within the same row with different superscripts are significantly different ($P < 0.05$)

The screening of the 5 isolates of *L. acidophilus* showed that each one could assimilate cholesterol from MRS broth over a period of 24 h (Table 2), but variation between the isolates was significant ($p < 0.05$); the isolate from the duodenum removed the most cholesterol (85.1%) and the isolate from the crop the least (65.0%). Significant differences with respect to tolerance of bile salts and degree of adhesion to the intestinal wall of the sheep were revealed also, but there was no apparent correlation between any of characteristics (13). As no single strain showed overwhelming superiority, it was decided to employ a combination culture that should, based on their comparative growth rates in milk, include approximately equal numbers of each isolate; facilities were not available to confirm that this anticipated balance was achieved in practice.

3.1 *Effect of treatment on milk yield*

The yield of milk/ewe over the period of 60 d after weaning ranged between 27.0 and 77.2 kg with an average of 42.9±4.2 kg for the treated group, compared with a spread of 14.0 to 53.4 kg with an average of 34.7±3.4 kg for the control group. However, the variation between individual ewes meant that there was no significant difference between the means, even though the overall yield of the treated group was ~20% above that of the control group.

3.2 *Effect of treatment on plasma and milk cholesterol levels*

The weekly/biweekly group means for cholesterol in 100 ml blood plasma ranged between 46.1 and 52.7 mg with an average of 48.7±0.53 mg for ewes in the treated group compared to a range of 49.5 to 54.6 mg with an average of 51.8±0.50 mg for ewes in the control group. The overall patterns are shown in Fig. 1 and, in both cases, there was a downward trend following lambing. The difference between the group means was not significant.

The group means for cholesterol levels per litre of milk ranged between 87 and 161 mg with an average of 124±6.6 mg for ewes in the treated group compared to a range of 149 to 182 mg with an average of 165±3.4 mg in the control group. The contrast between the groups is further highlighted in Fig. 2, and the overall mean level of cholesterol in the milk from the treated ewes was significantly ($p < 0.05$) lower than the figure recorded for the control group.

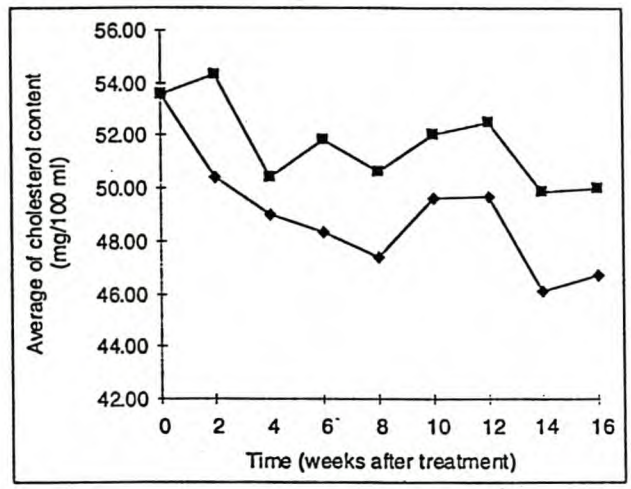

Fig. 1: The average cholesterol contents (mg/100 ml) of the blood sera of ewes (15/group) fed 2 capsules/d containing a total of 1.0×10^9 colony-forming units of *L. acidophilus* (◆ treated group) or no supplements (■ control group

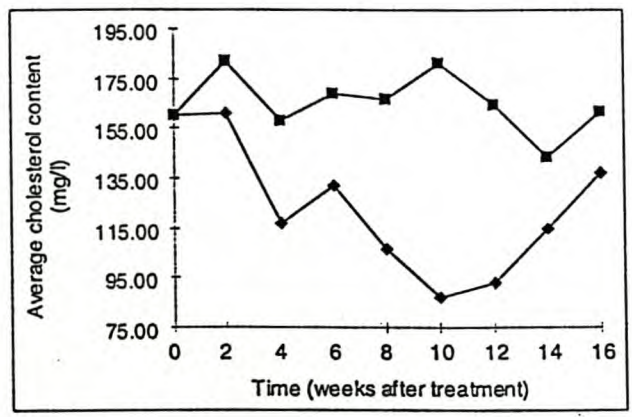

Fig. 2: The average cholesterol contents (mg/l) of milk from ewes (15/group) fed 2 capsules/d containing a total of 1.0×10^9 colony-forming units of *L. acidophilus* (◆ treated group) or no supplements (■ control group)

4. Discussion

Although biological variation between strains of *L. acidophilus* is not unusual, it was notable, in the present study, that while the isolate from the intestine was the most tolerant of bile salts, that from the crop showed the greatest ability to adhere to the tissue of the sheep intestine. This pattern suggests that the isolates may

have been subject to an element of natural selection by habitat, in that: (i) those surviving in the crop would benefit from the ability to adhere firmly to the host tissues; (ii) those in the duodenum would be exposed to the highest levels of dietary cholesterol; and (iii) those in the intestine would benefit from a high degree of tolerance to bile salts, but whether or not these apparent associations have any further physiological/morphological bases was not examined.

The apparent increase in milk yield as a result of supplementation was more dramatic than anticipated and, while it was not statistically significant, a stronger response might have been achieved had not the average milk yield for both groups been much lower than normal for the breed and location. Thus, the average for the Station is around 70 kg/ewe over 60 d and, given a more liberal flow of milk, the impact of supplementation with *L. acidophilus* could be interesting.

A decrease in serum cholesterol would be expected if the isolates of *L. acidophilus* have the ability to assimilate cholesterol in the lumen of the intestine but, in the present trial, no significant contrast with the control group was recorded (see Fig. 1). Nevertheless, supplementing the diet of milking ewes with *L. acidophilus* did result in a mean reduction of ~25% in the cholesterol content of their milk compared with the ewes in control group (see Fig. 2).

The dietary implications for humans could be important if the alleged link between dietary cholesterol and serum cholesterol levels is valid. Thus, most milk from the Awassi sheep is turned into Nabulsi cheese, which has, on average, a fat content of 300–310 g/kg (14). If the total cholesterol content of the milk passes into the cheese, then cheese made from the control milk might contain 0.78 g/kg of cheese, as compared to 0.59 g/kg for cheese made from milk from the treated flock. This reduction could be important in a country like Jordan where Nabulsi cheese is consumed in quantity with bread, in salads or as a basis for desserts like kunafa. Whether the cholesterol content of sheep milk could be reduced in practice remains to be established, but it would seem fair to conclude that the idea merits serious consideration.

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6. Summary

HADDADIN, M.S.Y., LUBBADEH, W., AL-TAMIMI, M.A., ROBINSON, R.K.: **A preliminary appraisal of the effect on the cholesterol content of fresh ovine milk of supplementing the feed of Awassi ewes with *Lactobacillus acidophilus*.** Milchwissenschaft 54 (9) 502–505 (1999).

38 Ovine milk (cholesterol content and feeding)

Although the cholesterol content of ovine milk is not high, when the milk is made into semi-hard, white brined cheeses, the level may reach 0.7–0.8 g/kg. In the Middle East, these cheeses can provide a major source of dietary cholesterol. Assuming that it is desirable for humans to lower their cholesterol intake, reducing the level in ovine milk/cheese could prove beneficial. This aim was achieved by supplementing the feed of Awassi ewes with selected strains of *Lactobacillus acidophilus*, and the mean cholesterol content in the milk was reduced by ~25%. If the same effect could be achieved on a commercial scale, the low cholesterol milk might both attract a premium price in the market place and help to reduce the cholesterol intake of local consumers.

HADDADIN, M.S.Y., LUBBADEH, W., AL-TAMIMI, M.A., ROBINSON, R.K.: **Vorläufige Bewertung der Wirkung eines Zusatzes von *Lactobacillus acidophilus* zur Fütterung von Awassi-Schafen auf den Cholsteringehalt von frischer Schafmilch.** Milchwissenschaft 54 (9) 502–505 (1999).

38 Schafmilch (Cholesteringehalt und Fütterung)

Obwohl der Cholsteringehalt von Schafmilch nicht hoch ist, wenn die Milch zu gesalzenem weißen Schnittkäse verarbeitet wird, kann er doch 0,7 bis 0,8 g/kg erreichen. Im Mittleren Osten können diese Käse daher für größere Mengen dietätischen Cholesterins sorgen. Nimmt man an, daß es für Menschen wünschenswert ist, die Cholesterinaufnahme zu reduzieren, könnte sich ein Senken in Milch/Käse als günstig erweisen. Dieses Ziel wurde erreicht, indem dem Futter von Awassi-Schafen ausgewählte *Lactobacillus acidophilus*-Stämme zugesetzt wurden. Der durchschnittliche Cholesteringehalt in Milch wurde um ~25% gesenkt. Könnte dieselbe Wirkung auf kommerzieller Basis erreicht werden, könnte Milch mit einem niedrigen Cholesteringehalt einen höheren Preis im Verkauf erreichen und helfen, die Cholesterinaufnahme der Konsumenten zu reduzieren.

SURVIVAL OF *LACTOBACILLUS ACIDOPHILUS* IN FERMENTED PRODUCTS

by

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ABSTRACT

The alleged 'health-promoting' properties of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* suggest that these species could be employed with advantage by the dairy industry, and the manufacture of two potentially suitable products is outlined. One — Acidophilus Yoghurt — contains *L. acidophilus* together with a normal yoghurt culture, but the other — a commercial product, Cultura — is fermented with selected strains of *L. acidophilus* and *B. bifidum*. Survival of the desired species throughout the expected shelf-lives of the products is assured.

Introduction

It has long been accepted that certain lactic acid bacteria can survive passage through the human digestive system, and can become implanted on the walls of the ileum (Nahaisi, 1986). In this position, it is believed that the bacteria secrete lactic acid, and perhaps antibiotic compounds as well, and in so doing, promote a healthy microflora in the large intestine. To what extent this change is beneficial is the subject of some debate, but there is evidence that consumers are becoming increasingly aware that fermented milks may be more than "just pleasant to eat". In Japan, for example, the sales figures for yakult are climbing rapidly, and in part, because consumers have been convinced about the health promoting attributes of the organism responsible for the fermentation — *Lactobacillus casei* (Lang, 1980).

In Europe and America, however, interest in *L. casei* has been muted by the fact that many strains secrete the nutritionally suspect D(−) lactic acid in excess of the acceptable L(+) isomer (Amer & Lammerding, 1983), and hence attention has centred on two other organisms, namely *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Both these organisms are claimed to possess health promoting and/or therapeutic properties, and among the various reports are included:

(i) suppression of certain types of tumour cells;
(ii) alleviation of habitual constipation, particularly in elderly or bed-ridden patients;
(iii) depression of serum blood cholesterol levels; and
(iv) restoration of a normal intestinal flora after antiobiotic treatment,

and other effects have no doubt been recorded (Nahaisi, *loc. cit;* Rasic and Kurman, 1983). The importance of these desparate claims, and their significance to any given individual, are matters for further study, but equally relevant is the problem of providing potential consumers with:

(i) the minimum daily dosage of the desired species; and
(ii) a product that is both organoleptically acceptable, and capable of maintaining the viability of the selected organism throughout the anticipated shelf-life of the retail item.

There are,of course, a number of solutions that might prove appropriate, but it is the employment of fermented dairy products as vehicles for the bacteria that forms the subject of this present study.

Acidophilus Products

A number of snack foods, such as sweets and jellies, have been proposed as carriers for *L. acidophilus*, but the viability of the organism has usually left much to be desired (Speck, 1980). The direct incorporation of the bacterium into non-fermented dairy products has proved more successful in microbiological terms, and Speck (*loc.cit.*) has indicated that *L. acidophilus* added to pasteurised milk will remain viable for 2-3 weeks at low temperature. A somewhat similar suggestion has been put forward by Klupsch (1983), who proposed that both *L. acidophilus* and *B. bifidum* could be introduced into a diet using butter or ice cream as carriers; no data are available concerning survival in these latter products.

Despite the obvious simplicity of the incorporation route, the derivation of an attractive fermented product could offer several advantages, such as:

(i) exploitation of the metabolic capabilities of the organisms in respect of, for example, flavour compounds;
(ii) delivery to the consumer of organisms that are physiologically active, so increasing, perhaps, the chances of successful inplantation; and
(iii) an opportunity to expand the range of fermented dairy products on retail sale.

These aims are not, of course, in themselves novel, but in the past, the available strains of *L. acidophilus* and *B. bifidum* were not readily amenable to commercial operations. However, the present generation of cultures are entirely predictable in their behaviour, and hence the only real obstacle to exploitation is their sensitivity to acid conditions. This characteristic is relevant in respect of their survival during the storage and distribution of any retail product, because if *L. acidophilus*, for example, is to have any therapeutic impact, it is usually suggested that the minimum level must be 1 x 10^5 viable cells per g of retail product (Nahaisi, *loc.cit.*). Attainment of such figures can be achieved either by maintaining a low acidity in the product, or by having such a high count initially that natural mortality still leaves the product with the therapeutic minimum at the end of its expected shelf-life. Either approach can achieve its desired goal, and the two processes described below support the validity of this conclusion.

Acidophilus Yoghurt

The widespread popularity of yoghurt would suggest that it could be employed as a vehicle for other lactic acid bacteria, but some reports indicate that *L. acidophilus* cannot survive in this medium (Gilliland & Speck, 1977). The overall acidity may be one of the reasons for these failures, but it has been suggested also that some strains of *Lactobacillus bulgaricus* — one of the usual starter organisms in yoghurt — can produce levels of hydrogen peroxide that are inhibitory to a susceptible species of bacterium (Tamine and Robinson, 1985).

In order to avoid these problems, the acidity of the "yoghurt" produced during this study was restrained at 0.6-0.7% lactic acid, and although the overall rate of inoculation was 2%, the yoghurt culture represented only 25% of the volume employed. The procedure used to produce the acidophilus yoghurt, in this case a drinking variant, is shown in Figure 1, and it was noticeable that:

TABLE 1 Survival of *L. acidophilus* in different Acidophilus Yoghurt drinks incorporating 2% butterfat and stored at 5°C.*

Storage Time (days)	Natural	Natural + 6% sucrose	Sweetened and Flavoured
Initial	95 x 10^5	67 x 10^5	77 x 10^5
7	76 x 10^5	49 x 10^5	46 x 10^5
14	40 x 10^5	24 x 10^5	26 x 10^5

* Means of duplicate samples — enumeration employing the technique of Hull & Roberts (1984).

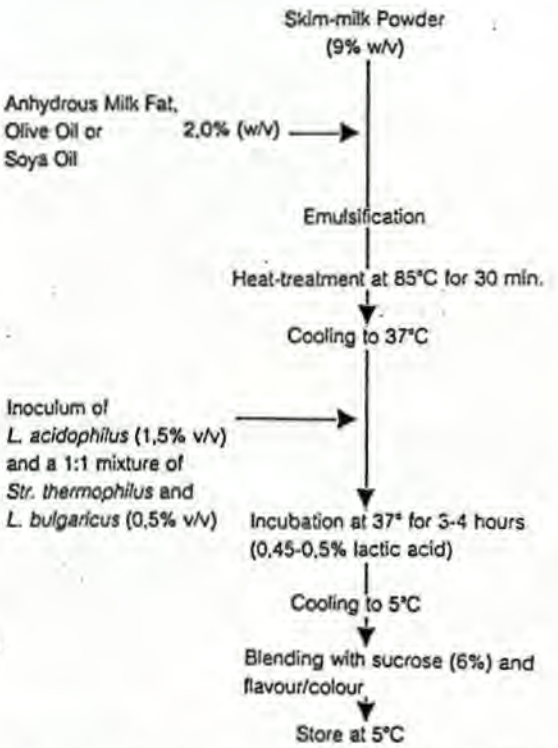

FIGURE 1 A simple procedure for the production of an Acidophilus Yoghurt Drink (Nahaisi & Robinson, 1985).

(a) the mixed culture produced the desired acidity rapidly compared with the action of *L. acidophilus* alone, i.e. 3-4 hours as against 10-12 hours;
(b) the end-product was accepted as a refreshing drink when flavoured with orange or pineapple and 6% sucrose; and
(c) the incorporation of the vegetable oils had no significant effect on flavour, and no separation of the lipid fractions was observed during storage at 5 °C for 14 days.

Equally important was the fact that *L. acidophilus* retained its viability during storage, and some typical results are shown in Table 1. Whether this survival is a reflection of the low numbers of *L. bulgaricus* employed, or of the inability of the strain involved to generate hydrogen peroxide is not clear but either way, the yoghurt drink proved to be an excellent carrier for *L. acidophilus*.

'Cultura' — an AB-milk product

If it is desirable to include *L. acidophilus* (A) in a diet, then the concurrent introduction of *Bifidobacterium bifidum* (B) could confer, perhaps, an additional benefit on the consumer (Schuler-Mayloth *et al.*, 1969), and the so-called Bioghurt and Biogarde products seek to exploit this potential (Klupsch *loc. cit.*). However, the present disadvantage with these products is that separate bulk starters have to be prepared for each organism, and hence there is considerable interest in Europe in the introduction of "Cultura".

In essence, it is a product with the consistency and flavour of a natural, stirred yoghurt, and indeed the method of manufacture is virtually identical with the standard yoghurt process. However, there are some essential differences from the procedure outlined for the production of Acidophilus Yoghurt, and in particular:

(i) the anticipated cell counts of 2-4 x 10^8/ml (*L. acidophilus*) and 1-2 x 10^8/ml (*B. bifidum*) in the final product are achieved through the addition of cultures with high numbers of viable bacteria;
(ii) the product reaction of pH 4,1-4,2 is lower than can be accepted normally in an Acidophilus product, because the inoculation rate is such that the decline in cell numbers is accommodated with ease; and
(iii) the concentrated cultures of 'A' and 'B' are offered for Direc-to-Vat inoculation, so that the tedium of maintaining these fastidious species is avoided entirely (Anon, 1985).

It is clear, therefore, that the advent of the 'AB' cultures has extended the possible routes for incorporating desirable bacteria into a diet. Obviously the debate concerning the alleged therapeutic value of *L. acidophilus* and *B. bifidum* will continue, but if their consumption is deemed desirable, then either Acidophilus Yoghurt or Cultura could prove to be attractive vehicles. The advantage of the yoghurt process is that it is more versatile in relation to the range of cultures that could be explored, but it suffers from the major drawback that the levels of acidity are so critical. It is probably for this reason that Cultura has provoked a favourable reaction (Hansen, 1985), for in addition to providing an additional organism of potential therapeutic benefit, the procedure for manufacture is not only extremely straightforward, but also ensures that the product retains a high level of viable organisms. The extent to which consumers will become willing to buy products on the basis of a 'health-promoting' image remains to be seen, but should the demand arise, at last production is now a feasible proposition.

Acknowledgements

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REVIEW ARTICLE

Microbiological and technological aspects of milks fermented by bifidobacteria

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CONTENTS

INTRODUCTION AND HISTORICAL BACKGROUND

Ever since Metchnikoff (1910) first put forward the idea that the regular consumption of fermented milks might offer certain benefits with respect to the health of the consumer, the possible prophylactic and/or therapeutic properties of yogurt and related products have been the subject of much speculation. Certainly,

the Russians have long advocated the administration of kefir and koumiss to patients with a wide range of illnesses (Koroleva, 1991), but the varying microbiology of these products has made it difficult to confirm any theoretical basis for the claims. In the USA, acidophilus milk manufactured with *Lactobacillus acidophilus* was promoted in much the same manner, and whilst the concept equating consumption of 'acidophilus' products with 'good health' was regarded as entirely credible, poor quality control with respect to the microbiological and organoleptic properties of the retail items allowed the idea to fall into disrepute.

At present, lactic acid bacteria (i.e. mesophilic and thermophilic organisms) are widely used for the production of cheese and fermented milks including yogurt (Marshall, 1987; Tamime & Robinson, 1988; Tamime, 1990). However, in recent years new fermented dairy products have been developed and marketed in Europe, North America and the Far East that:

> include *Lb. acidophilus*, *Bifidobacterium* spp., *Lb. casei* subsp. *casei* biovar *shirota* and *Lactobacillus* strain GG similar to *Lb. casei* subsp. *rhamnosus* (Dong *et al.* 1987) and/or combinations of these amongst the bacteria in the starter culture;
> are expected to contain species/strains of bacteria that were isolated originally from humans;
> and have $> 10^6$ viable cells of each culture ml^{-1} product at the time of consumption.

The longstanding interest in the potential 'health-promoting' properties of *Lb. acidophilus* is well documented (Gilliland, 1989; Gilliland & Walker, 1990; Sellars, 1991; Salji, 1992, 1993), but possible role(s) of ingested bifidobacteria have been subject to less intense scrutiny (Robinson & Samona, 1992). Nevertheless, the importance of the bifidobacteria with respect to the healthy operation of the human digestive system is not in doubt, and hence it is entirely appropriate to evaluate the contention that the consumption of 'bifidus' products should be encouraged. In particular, this review will consider: (i) the characteristics of the genus *Bifidobacterium*, (ii) the ecology of the genus within the human intestine and (iii) the potential for manufacturing dairy products that will act as carriers for selected species.

NOMENCLATURE AND CLASSIFICATION

A wide range of microorganisms can colonize the human intestine where some are beneficial and others are undesirable. By the turn of this century, one of the beneficial bacteria had been isolated and identified by Tissier (1900); it was designated *Bacillus bifidus*. Between then and the 1950s (Bergey's Manual of Determinative Bacteriology, 7th edn), Tissier's designation was changed and other generic names were proposed, but the generic name most commonly used was *Lactobacillus bifidus* (Poupard *et al.* 1973; Rašić & Kurmann, 1983).

In the 1970s only eleven bifidobacteria were identified, and they were grouped in the family Actinomycetaceae which in turn consists of five genera (Rogosa, 1974). Recently, Scardovi (1986) and Biavati *et al.* (1992) grouped twenty-four different species of *Bifidobacterium* separately as 'irregular, non-sporing and Gram-positive rods'. These organisms were isolated from different sources such as: (i) the faeces of humans (infants and adults), animals, birds and sewage, (ii) bees, (iii) the human vagina and (iv) dental caries, the last being considered as pathogens. At present, only five species of *Bifidobacterium* have attracted attention in the dairy industry for the

Table 1. *Current nomenclature of some* Bifidobacterium *spp. relevant for the manufacture of fermented milk products*

New	Old
Bifid. adolescentis	*Bifid. adolescentis*
Bifid. bifidum	*Bacillus bifidus communis* *B. bifidus* *Bacteroides bifidus* *Bacterium bifidum* *Tissieria bifida* *Nocardia bifida* *Actinomyces bifidus* *Actinobacterium bifidum* *Bifid. bifidum* *Lactobacillus bifidus* *Lb. parabifidus* *Cohnistreptothrix bifidus* *Actinomyces parabifidus*
Bifid. breve	*Bifid. parvulorum*
Bifid. infantis	*Bifid. liberorum* *Bifid. lactentis* *Bifid. parabifidum*
Bifid. longum	*Bifid. longum* subsp. *animalis*

After Rogosa (1974). Rašić & Kurmann (1983), Scardovi (1986) and Puhan (1988).

manufacture of therapeutic fermented milk products. In this review, only the indigenous microflora of the human will be discussed, and the historical taxonomy of bifidobacteria, including the latest nomenclature is shown in Table 1.

TAXONOMIC INFORMATION

According to Rašić & Kurmann (1983), Mitsuoka (1984, 1992), Scardovi (1986), Modler *et al.* (1990*a*). Biavati *et al.* (1992) and Ballongue (1993), the differentiating characteristics of *Bifid. bifidum*, *breve*, *longum*, *adolescentis* and *infantis* could be summarized as follows.

Morphology

The cell morphology of *Bifidobacterium* spp. grown anaerobically in stabs of trypticase–phytone–yeast extract medium showed that some bifidobacteria had distinctive cellular shapes and arrangements. These traits could be summarized as:

grouping of 'amphora-like' cells (*Bifid. bifidum*),
specific epithet, thinnest and shortest cell among bifidobacteria (*Bifid. breve*) and
very elongated, relatively thin cells with slightly irregular contours and rare branching (*Bifid. longum*).

Bifid. adolescentis and *infantis* have cellular morphology that is nonspecific and similar to that of many other species of bifidobacteria; Fig. 1 shows some examples of cellular morphology in the genus *Bifidobacterium*.

Cell wall structure

A summary of the cell wall constituents of *Bifid. adolescentis*, *bifidum*, *breve*, *infantis* and *longum* is shown in Table 2, and the principal component is peptidoglycan (murein). This complex material consists of linear chains of a

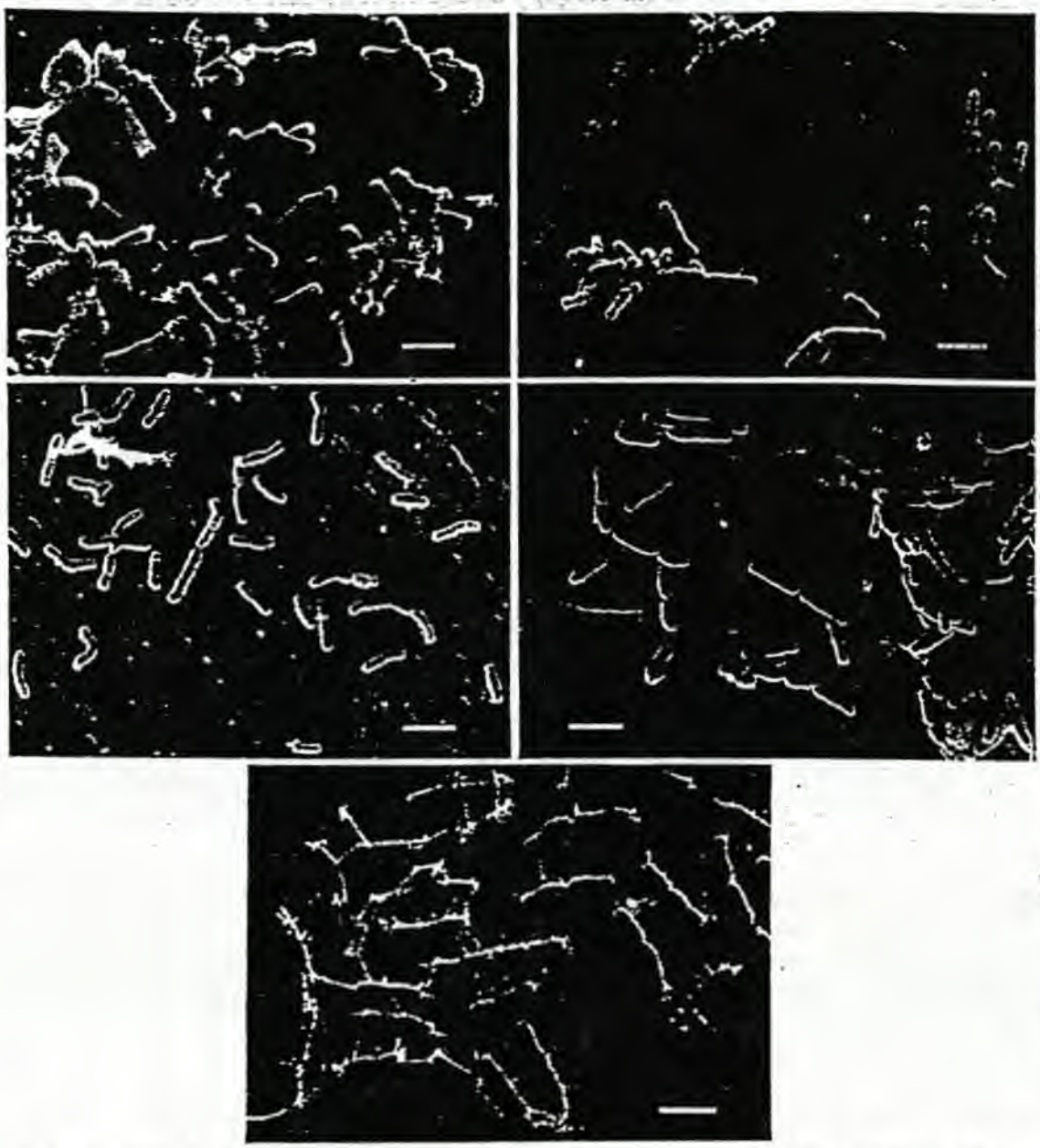

Fig. 1. Cellular morphology of five different species of *Bifidobacterium*: (*a*) *Bifid. adolescentis* ATCC 15703, (*b*) *Bifid. bifidum* ATCC 29521, (*c*) *Bifid. breve* ATCC 15700, (*d*) *Bifid. infantis* ATCC 15697, (*e*) *Bifid. longum* ATCC 15707. Bar size, 2 μm. *Cultivation*. MRS broth (Institute Rosell Inc., Montreal, Canada) supplemented with (g/l): L-cysteine hydrochloride, 0·5; Tween 80, 1: filter-sterilized glucose, 20 was used to rehydrate the freeze-dried microorganisms and recovered strains were subcultured twice. Active cultures were incubated at 37 °C for 15 h in an anaerobic chamber (Forma Scientific, Marietta, OH, USA) with an atmosphere of (%): CO_2, 5; H_2, 10; N_2, 85. *Scanning electron microscopy*. Specimens of bifidobacteria (3 ml suspensions) were deposited on a 0·22 μm filter and all subsequent steps were carried out with a 5 ml syringe. Fixation was with glutaraldehyde (30 g/l)–cacodylate (0·1 M, pH 7·3) for 1·5 h at room temperature and was followed by a 1 h rinse in cacodylate buffer. Dehydration was carried out with graded ethanol series. Samples were critical point dried, covered with gold and examined using a Nanolab LE2100 at 5 kV. (After D. Roy & D. Montpetit, pers. comm.) Reproduced by courtesy of the Food Research and Development Center, Canada.

polysaccharide composed of molecules of *N*-acetylmuramic acid and *N*-acetylglucosamine alternating along the length of the chain (Ballongue, 1993). These chains are crosslinked by tetrapeptides consisting of alanine, glutamic acid and ornithine or lysine, while links between adjacent tetrapeptides may consist of one or

Table 2. *Cell wall composition of some selected species of bifidobacteria*

Species	Type of peptidoglycan or murein	Polysaccharides		
		Glucose	Galactose	Rhamnose
Bifid. adolescentis	Lys or Orn-D-Asp	+	+	−
Bifid. bifidum	Orn or Lys-D-Ser-D-Asp	+	+	+
Bifid. breve	Lys-Gly	+	+	+
Bifid. infantis	Orn or Lys-Ser-Ala-Thr-Ala	+	+	+
Bifid. longum	Orn or Lys-Ser-Ala-Thr-Ala	+	+	+

Compiled from Rašić & Kurmann (1983), Scardovi (1986) and Ballongue (1993).

Table 3. *Carbohydrate fermentation characteristics for differentiating the species of* Bifidobacterium

	Bifidobacterium spp.				
	adolescentis	*bifidum*	*breve*	*infantis*	*longum*
Arabinose	+	−	−	−	+
Cellobiose	+	−	(+)	−	−
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Gluconate	+	−	−	−	−
Inulin	(+)	−	(+)	(+)	−
Lactose	+	+	+	+	+
Maltose	+	−	+	+	+
Mannitol	(+)	−	(+)	−	−
Mannose	(+)	−	+	(+)	(+)
Melezitose	+	−	(+)	−	+
Melibiose	+	(+)	+	+	+
Raffinose	+	−	+	+	+
Ribose	+	−	+	+	+
Salicin	+	−	+	−	−
Sorbitol	(+)	−	(+)	−	−
Starch	+	−	−	−	−
Sucrose	+	(+)	+	+	+
Trehalose	(+)	−	(+)	−	−
Xylose	+	−	−	(+)	(+)

+, Positive reaction by 90% or more strains; −, negative reaction by 90% or more strains; (+), positive reaction, but ferment slowly.

After Scardovi (1986) and Biavati *et al.* (1992).

more of the amino acids glycine, serine, aspartic acid and threonine. The amino acid composition of the basic tetrapeptides (see Table 2) can vary between species and/or strains of the same species, as can their sequence in the chain. Strain differences can arise also from the substitution of ornithine by lysine, and the extent of crosslinkage between chains, i.e. crosslinks may arise between one, two or three amino acids in adjacent sequences (Rašić & Kurmann, 1983).

Glucose, galactose and rhamnose are usual components of the cell wall structure, with qualitative and quantitative differences being observed with respect to species, strain and growth medium (Table 2). The principal fatty acids are myristic, palmitic, palmitoleic and oleic, but the precise composition varies considerably with growth medium, the presence or absence of human milk and growth temperature (Biavati *et al.* 1992). The phospholipids, such as phosphatidylglycerol and diphosphatidylglycerol, are well represented (Habu *et al.* 1987), along with derivatives like alanylphosphatidylglycerol which are specific to bifidobacteria (Răsić & Kurmann, 1983). Lipoteichoic acids, which appear to be essential for cell adhesion to the wall

Table 4. *Electrophoretic patterns of enzymes of some bifidobacteria*

Species	Transaldolase	6-Phosphogluconic dehydrogenase (NADP+)†	Fructose-6-phosphate phosphoketolase‡
Bifid. adolescentis	8	5	15
Bifid. bifidum	7	7–(8)	15
Bifid. breve	6	(5)–6–6–7	15
Bifid. infantis	(5)–6–8	3–4–(5)	15
Bifid. longum	(5)–6–8	5–(6)	15

†Numbers 1–10 and 1–13 are given to isozymes of transaldolase and 6PGD respectively in the order of decreasing anodic mobility.

‡ Number indicates the migration relative to that of *Bifidobacterium globosum* = 10 (i.e. ecologically is 'animal' type) and 15 is distinguished as of 'human' origin.

Numbers in parentheses are isozymes found in < 10% of strains studied; numbers in bold represent the isozyme of the type strain.

After Scardovi (1986).

of the intestine, form links with the polysaccharide chains but, once again, the precise nature of these acids is not constant.

Carbohydrate utilization

The ability of microorganisms to metabolize different types of carbohydrates has been used for identification purposes, and Table 3 illustrates the situation among *Bifidobacterium* spp. It is evident that *Bifid. adolescentis* can utilize a wide range of carbohydrates, followed by *Bifid. breve*, *infantis* and *longum*. However, *Bifid. bifidum* can only utilize fructose, galactose and lactose. Nevertheless, all five species of *Bifidobacterium* can grow in milk because they are able to ferment lactose as their source of energy. Recently, Roy & Ward (1990) have proposed a rapid gas chromatographic method for the quantitative evaluation of carbohydrate utilization (see also Roy *et al.* 1991*a*, *b*).

One of the key enzymes, which has been identified as typical of bifidobacterial hexose metabolism, is fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22). This enzyme is present in cellular extracts, and the fermentation of carbohydrates through this particular metabolic pathway is known as the 'bifid shunt'. Although F6PPK has been identified as the key enzyme, three types were detected by starch gel electrophoresis, and Table 4 illustrates these differences.

There are, however, strain differences in carbohydrate utilization (Table 5), and in one laboratory (SAC) we have found some discrepancies among *Bifid. longum* BF1 and *Bifid. infantis* NCFB 2205. For example, both organisms are able to ferment mannose when tested using API 50 CH galleries. Furthermore, although inulin is generally not utilized, there are reports (Yazawa *et al.* 1978; McKellar & Modler, 1989) that some intestinal species exhibit inulinase (EC 3.2.1.7) activity.

Urease activity

Urease (EC 3.5.1.5) activity in 'non-human' *Bifidobacterium* spp. is high, and the strongest ureolytic activity has been detected in *Bifid. suis* (> 80% of strains studied) (Crociani & Matteuzzi, 1982). However, *Bifid. bifidum* is weakly ureolytic, and only 10% of strains of *Bifid. breve* and *longum* studied have urease activity.

Deoxyribonucleic acid (DNA) homology

The guanine plus cytosine molecular percentage of the DNA of the genus *Bifidobacterium* is 58 for the human strains based on the buoyant density. The rest of the species have guanine plus cytosine ranging between 55 and 66% based on

Table 5. *Carbohydrate fermentation by certain species of bifidobacteria over 24 or 48 h*

Bifidobacterium spp.		Carbohydrates															
		Amygdalin		Aesculin		Fructose		Galactose		Gentibiose		Glucose		Lactose		Maltose	
		24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
adolescentis	2204 NCFB†	⁀	+	~	⁀	−	+	~	+	−	⁀	⁀	+	⁀	+	+	+
bifidum	2715 NCFB	~	+	~	+	~	+	⁀	+	−	⁀	+	+	+	+	+	+
	2203 NCFB	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
	XH‡	~	⁀	+	+	−	−	−	−	−	⁀	~	⁀	−	−	+	+
	Bb12§	−	−	−	−	+	+	⁀	⁀	−	−	+	+	+	+	+	+
breve	2258 NCFB	−	−	−	−	~	+	~	+	−	−	~	+	~	+	⁀	+
infantis	2257 NCFB	−	−	−	−	+	+	~	~	−	−	+	+	+	+	⁀	⁀
	2205 NCFB	−	−	−	−	+	+	⁀	⁀	−	−	+	+	+	+	⁀	⁀
	420‖	~	+	~	⁀	−	−	−	−	−	⁀	+	+	~	⁀	+	+
longum	2716 NCFB	−	−	−	−	−	−	~	⁀	−	−	⁀	+	~	+	⁀	+
	BF1¶	−	~	+	+	~	+	−	−	−	−	~	+	~	+	−	+

Bifidobacterium spp.		Carbohydrates													
		Melibiose		Raffinose		Ribose		Saccharose		Trehalose		Turanose		D-xylose	
		24	48	24	48	24	48	24	48	24	48	24	48	24	48
adolescentis	2204 NCFB†	⁀	+	+	+	−	−	+	+	−	−	−	−	−	−
bifidum	2715 NCFB	+	+	+	+	−	−	+	+	−	−	~	⁀	−	⁀
	2203 NCFB	−	−	−	−	−	−	−	−	−	−	−	⁀	−	−
	XH‡	+	+	~	⁀	−	~	⁀	⁀	−	−	−	~	−	−
	Bb12§	−	−	−	−	−	−	+	+	⁀	+	−	−	−	−
breve	2258 NCFB	~	+	~	+	−	−	−	−	−	−	−	~	−	−
infantis	2257 NCFB	−	−	−	−	−	−	+	+	−	−	−	−	−	−
	2205 NCFB	−	−	−	−	−	−	+	+	−	−	−	−	−	−
	420‖	+	+	+	+	~	+	+	+	−	−	~	⁀	−	−
longum	2716 NCFB	⁀	+	⁀	+	−	−	~	+	−	−	−	−	~	~
	BF1¶	−	+	~	+	−	−	−	+	−	−	−	−	−	−

−, Negative reaction; ~, positive reaction (50 %); ⁀, positive reaction (60–90 %); +, positive reaction (100 %).

Cultures were obtained from: † National Collection of Food Bacteria (formerly known as National Collection of Dairy Organisms, NCDO), ‡ New Zealand Dairy Research Institute (mutant strain from Chr. Hansen's Laboratorium), § Chr. Hansen's Laboratorium A/S, ‖ Laboratorium, Visby Tønder aps and ¶ RP Group Lacto-Labo.

After A. Y. Tamime (unpublished results).

Table 6. *Deoxyribonucleic acid (DNA) homology relationships (%) among the human strains of bifidobacteria*

	Bifidobacterium spp.				
Microorganism	*adolescentis*	*bifidum*	*breve*	*infantis*	*longum*
Bifid. adolescentis	70–102	14	5–10	22	
Bifid. bifidum	0	100	10–20	20–28	2
Bifid. breve		25	100	40	40
Bifid. infantis		42	17–50	74–101	50–79
Bifid. longum	0	40	12	50–76	75–101

Values in bold print represent most closely related species.
After Scardovi (1986).

melting temperature. The relatedness of DNA homology of human type bifidobacteria is shown in Table 6. The two species that are closely related are *Bifid. infantis* and *longum* (Lauer & Kandler, 1983; Scardovi, 1986; Imamura *et al.* 1990; Yaeshima *et al.* 1992).

Miscellaneous characteristics

Some characteristics that could be used for group differentiation of bifidobacteria include: (i) none of these organisms is capable of reducing nitrate, indole formation, liquefaction of gelatin or fermentation of glycerol, (ii) to date plasmids have been found in only four species (*Bifid. longum, asteroides, globosum* and *indicum*) and possibly in *Bifid. breve* (Iwata & Morishita, 1989) and (iii) some antibiotics can inhibit the growth of bifidobacteria, including the human strains (Miller & Finegold, 1967; Scardovi, 1986; Khedkar *et al.* 1990; Miyazaki *et al.* 1991; Lim *et al.* 1993).

Some enzyme activities (e.g. α-galactosidase (EC 3.2.1.22) and α-glucosidase (EC 3.2.1.20)) present in bifidobacteria could be used for rapid differentiation and identification (Chevalier *et al.* 1990). The same authors reported that two bifidobacteria strains of the American Type Culture Collection (*Bifid. adolescentis* ATCC 11146, *Bifid. bifidum* ATCC 11147) and three *Bifid. bifidum* strains from Biogarde® products did not belong to the genus *Bifidobacterium* because the strains were α-glucosidase-negative, and F6PPK activity was not detected. Incidentally, α-galactosidase activity has not been detected in any of the eight *Lactobacillus* strains studied (see also Desjardins *et al.* 1990; Roy & Ward, 1992; Roy *et al.* 1992).

BIOCHEMISTRY AND PHYSIOLOGY OF BIFIDOBACTERIA

Introduction

Bifidobacteria that are to be selected for milk fermentations have as their target destination the 'normal' habitat of the large intestine and colon. The two environments are different. but the bifidobacteria are versatile organisms and are able to survive under different conditions of oxygen availability and to ferment a wide range of substrates, including lactose. In a healthy adult colon, bifidobacteria share a common habitat with other anaerobic bacteria such as the *Bacteroides, Veillonellae* and *Eubacterium*.

Anaerobic lactobacilli and bifidobacteria may be the dominant part of a faecal flora of 1×10^{10} cfu g^{-1}. It may be noted, however, that bifidobacteria also share some common characteristics of non-colonic types of lactobacilli and streptococci with regard to their ability to tolerate oxygen and their sugar fermentation patterns.

In an environment of even temperature and complex ecosystem, the physiology and biochemistry of this genus can be expected to be complex. The organisms do not grow below 20 °C and as a general rule there is no thermoresistance above 46 °C (Ballongue, 1993). *Bifid. bifidum* dies at 60 °C (Rašić & Kurmann, 1983). There is also a narrow optimum pH range for growth: pH 6·5–7·0 with no growth at pH < 5·0 or > 8·0 (Scardovi, 1986). Most species are unable to initiate growth and develop in chemically defined synthetic media (Petschow & Talbott, 1990, 1991).

Physiology

Bifidobacteria are classed as anaerobic organisms, although some species are able to tolerate oxygen. Generally, oxygen toxicity results from the effects of different 'activated' oxygen compounds such as superoxide and hydroxyl radicals and hydrogen peroxide. Two enzymes, superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6), are important in the defence against the toxic effects of superoxide and hydrogen peroxide, but these are absent or present only as a very low activity in *Bifid. infantis, breve, longum* and *adolescentis*. In addition, oxygen sensitivity in these strains varies independently of the superoxide dismutase activity (Shimamura *et al.* 1992). This would indicate the operation of other mechanisms. Oxygen tolerance is related to oxygen utilization. Even in the absence of exogenous carbohydrate, some strains utilize oxygen during metabolism of intracellular substrates (Shimamura *et al.* 1990). This may be of significance for their ability to maintain viability after fermentation and ingestion.

The degree of tolerance is influenced by the culture medium. De Vries & Stouthamer (1969) studied the effects of oxygen on a number of strains. Some strains grew in the presence of oxygen without accumulating hydrogen peroxide, while other strains exhibited limited growth and accumulated hydrogen peroxide. One strain appeared intolerant to oxygen and grew only when the redox potential was lowered: the presence of oxygen prevented growth because of the difficulty of establishing a suitable redox potential.

NADH oxidase (EC 1.6.99.3) and NADH peroxidase (EC 1.11.1.1) are likely to be the significant enzymes involved in oxygen utilization. These enzymes catalyse the reactions

$$O_2 + NADH + H^+ \rightarrow H_2O_2 + NAD^+$$

and

$$H_2O_2 + NADH + H^+ \rightarrow 2H_2O + NAD^+.$$

A coupling of these two enzymes will result in growth without accumulation of hydrogen peroxide. Should the enzymes not be coupled, i.e. because of mismatched activity or through lack of available NADH, the hydrogen peroxide cannot be completely removed. Growth may then be impaired because of hydrogen peroxide accumulation. The competition for NADH by these two enzymes and by lactate dehydrogenase (EC 1.1.1.27) which is balanced by the supply of the co-factor from glucose degradation (see below) may explain the behaviour of these bacteria in nutrient-limiting environments.

Carbohydrate metabolism

Bifidobacteria were classified into a new genus setting them apart from the *Lactobacillus* spp. as a consequence of taking into account other criteria including metabolic activity. In the genus *Bifidobacterium* hexoses are degraded by a fructose-6-phosphate shunt rather than a glucose-6-phosphate shunt which is used by the

heterolactic lactobacilli (Scardovi & Trovatelli, 1965; De Vries *et al.* 1967). This alternative phosphoketolase pathway (Fig. 2) appears to be specific to the genus *Bifidobacterium* and is characterized by the presence of F6PPK and, in common with the heterolactic lactobacilli, an absence of fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), which would commit the sugar to homolactic fermentation.

The fermentation of two moles of glucose leads to two moles of lactate and three moles of acetate if pyruvate is converted to lactate by an NADH-dependent lactate dehydrogenase. Unlike the hexose monophosphate shunt of the lactobacilli there is no carbon dioxide production.

There are, however, other fates for pyruvate: it may be split to yield acetate and ethanol by a phosphoroclastic split or may be broken down to formate and ethanol. There is no direct evidence, however, for the phosphoroclastic enzyme or for the pyruvate–formate lyase pathway. Circumstantial evidence from observations of actively growing cells, however, suggests that pyruvate must be directed to other end-metabolites as the theoretical ratio of fermentation (i.e. 2 hexose → 3 acetate + 2 lactate) could not be confirmed experimentally (Scardovi, 1986). In addition, a slight increase in uric and formic acids (2 μg g^{-1}) was reported by Mahdi (1990) when cows' and ewes' milks were fermented with *Bifid. bifidum*.

In the 1960s, Dutch workers (De Vries & Stouthamer, 1967, 1968, 1969; De Vries *et al.* 1967, 1970) reported the absence of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and aldolase, thus ruling out the glycolytic and monophosphate pathways. However, more recently, low but detectable levels of these enzymes have been found in some *Bifidobacterium* spp. (Scardovi, 1986; Biavati *et al.* 1992).

Nitrogen metabolism

Many strains of bifidobacteria are able to use ammonium salts as a nitrogen source (Hassinen *et al.* 1951), but others including *Bifid. suis* and *Bifid. cuniculi* require some organic nitrogen (Ballongue, 1993). *In vitro*, *Bifid. bifidum* produces alanine, valine, aspartate and threonine (Matteuzzi *et al.* 1978).

Some strains do not exhibit sufficient proteolytic activity for growth in milk. Milk contains few free amino acids and peptides. For rapid growth, milk has to be supplemented with casein hydrolysate (Klaver *et al.* 1993), a nutrient-rich broth (Collins & Hall, 1984; Desjardins *et al.* 1990) or yeast extract (Roy *et al.* 1990).

While there has been considerable interest in developing these organisms for milk fermentation, little work has been carried out on their proteinases and peptidases. El-Soda *et al.* (1992) have reported general caseolytic activity for *Bifid. infantis* and *longum*. Exopeptidases are produced by several species (Minagawa *et al.* 1985), and Cheng & Nagasawa (1985*a*, *b*) have purified an aminopeptidase (EC 3.4.11.11) and a proline iminopeptidase (EC 3.4.11.5) from *Bifid. breve*; carboxypeptidase (EC 3.4.17.1) activity has also been demonstrated (El-Soda *et al.* 1992).

It would appear that, for some strains, growth in milk is possible. However, there are conflicting reports: Klayer *et al.* (1993) found some strains unable to grow in milk while others (Desjardins *et al.* 1991) found that the same strains can grow in milk. Because of the inconsistency in growth in milk, mixed starters are often used for the production of fermented milks. Studies on the associative growth between lactobacilli and bifidobacteria in milk indicate a stimulation of the bifidobacteria due to proteolytic enzymes and aminopeptidase of the lactobacilli (Cheng & Nagasawa, 1983).

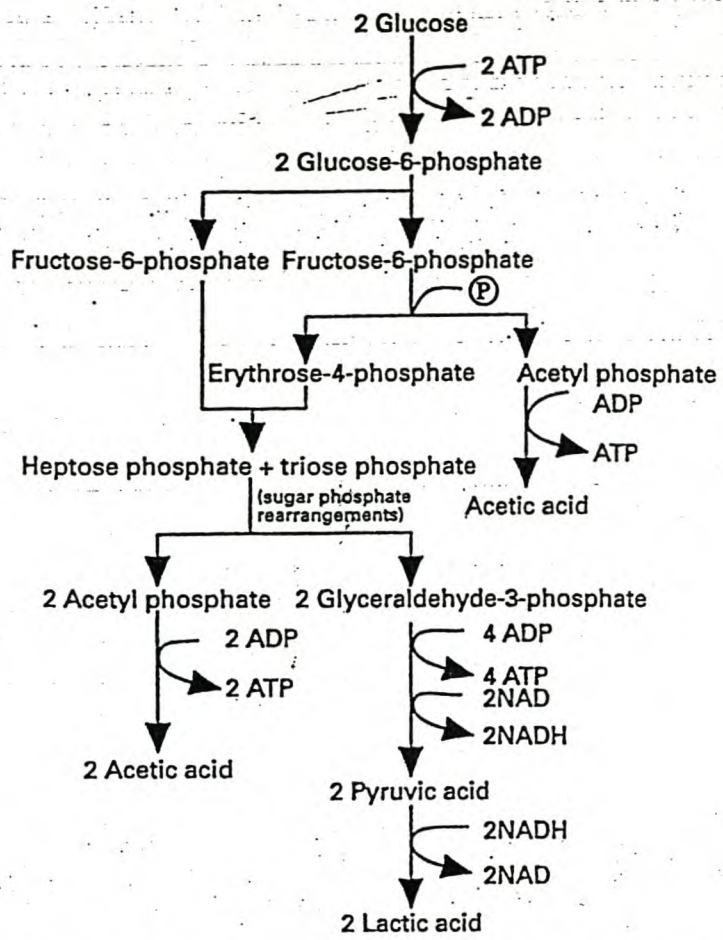

Fig. 2. Metabolic pathway of glucose fermentation by *Bifidobacterium* spp. (After Stanier *et al.* 1987.) Reproduced by courtesy of Macmillan Press Ltd.

Fat metabolism

No data are available on fat metabolism by the genus *Bifidobacterium*. However, when Greek-style yogurt was made by ultrafiltration using cows' and ewes' milks fermented with *Bifid. bifidum*, the increase in total fatty acid contents was marginal: 0·39 and 0·33 mmol KOH kg^{-1} respectively (Mahdi, 1990). It is possible that the bifidobacteria do not possess any lipase activity.

Growth factors

The efforts made to study bifidobacteria *in vitro* and to develop a milk containing large numbers of cells has resulted in the description of many growth promoting factors (Hidaka *et al.* 1990; Salyers, 1990; Harju, 1993; Smart, 1993; Tamura *et al.* 1993; Modler, 1994). Recently, Kaneko *et al.* (1994) have identified a bifidogenic growth stimulator for *Bifid. adolescentis* 6003 which was present in the cell-free filtrate of *Propionibacterium freudenreichii* 7025 and in the methanol extract fraction of the cells; however, short-chain fatty acids (formate, acetate, propionate and butyrate) also stimulated the growth of bifidobacteria.

Bifidus factor 1. This growth factor is a component of milk and colostrum. Originally described by György *et al.* (1954*a*), it can be separated into two factors by isocratic HPLC (Ashoor & Monte, 1983). It appears to consist of glycoproteins where the 'glyco' moiety contains an *N*-acetylglucosamine. Native human casein or

its trypsin derivative (Bezkorovainy *et al.* 1979) are effective promoters for *Bifid. bifidum*. Trypsin hydrolysis of human κ-casein results in a glycomacropeptide which contains sugars such as glucosamine and galactosamine, which are also active. Azuma *et al.* (1984) found that the glycomacropeptide from chymotrypsin hydrolysis of human κ-casein was more effective than the native κ-casein and promoted the growth of *Bifid. bifidum*. These authors also suggested that the sugar and the polypeptide were important to the activity of the bifidus factor.

Similar bifidus factors are found in mucus secreted by the salivary glands, the small intestine and the colon (Allen, 1984). These too contain glycoproteins with *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid components.

Bifidus factor 2. Glycoproteins from milk and from whey seem to be generally effective. However, 'bifidus factor 2' appears to be the non-glycosylated peptides of casein after hydrolysis using proteinase (György *et al.* 1954*b*).

Oligosaccharides. Oligosaccharides have also been studied extensively for their growth promoting ability. Human milk contains a variety of complex oligosaccharides which may be responsible for promoting the growth of *Bifid. bifidum*, the species which predominates in the faecal flora of the breast-fed infant (Beerens *et al.* 1980). The ability of bifidobacteria in general to utilize oligosaccharides has fuelled the search for oligosaccharides, natural and synthetic, that will not be utilized by other intestinal bacteria but will, on reaching the large intestine and colon, promote the growth of desirable bifidobacteria.

The naturally occurring raffinose (a trisaccharide containing galactose) and stachyose (a tetrasaccharide) are utilized by many species of bifidobacteria. The doubling time of *Bifid. infantis* grown in media containing raffinose and stachyose was similar to that obtained with glucose and lactose. *Bifid. bifidum*, however, does not utilize raffinose (Yazawa *et al.* 1978).

Fructo-oligosaccharides are polymers containing D-fructose terminating with an α (1 → 2) linked glucose. Oligosaccharides of three to five units are sometimes referred to as 'neo-sugars'. They occur naturally in the Jerusalem artichoke. Several species of bifidobacteria are able to utilize these sugars in laboratory media containing the neosugar as the only carbohydrate source (McKellar & Modler, 1989; Yamazaki & Matsumoto, 1994).

Synthetic oligosaccharides have also been investigated. The synthesis of transgalactosylated oligosaccharide has been patented (Japanese Patent, 1980; for other patents see Smart, 1993). This tetrasaccharide can be utilized by a number of bifidobacteria and by some lactobacilli and enterobacteria (Tanaka *et al.* 1983). Other synthetic oligosaccharides have also been studied for species specificity. Galsucrose and lactosucrose were utilized by the bifidobacteria and by 62 and 41% respectively of the 37 strains of enterobacteria tested. This compared with raffinose which was utilized by 47% of the enterobacteria (Minami *et al.* 1983). This specificity is not confined to the bifidobacteria.

Other higher molecular mass oligosaccharides may be useful. These are given the general term 'inulin'. Many species of bifidobacteria demonstrate inulinase activity (Yazawa *et al.* 1978; McKellar & Modler, 1989).

Synthesis of vitamins

Bifidobacterium strains of human origin seem to require thiamin (B_1), pyridoxine (B_6), folic acid (B_9) and cyanocobalamin (B_{12}), but the work of Deguchi *et al.* (1985) indicated that the same organisms are also capable of synthesizing some B group vitamins. Table 7 illustrates the production of such vitamins by *Bifid. adolescentis*,

Table 7. *Vitamin synthesis by and requirement of some species of bifidobacteria*

Vitamin	*Bifidobacterium* spp.				
	adolescentis	*bifidum*	*breve*	*infantis*	*longum*
Thiamin, $\mu g\ ml^{-1}$	0·02 (+)	0·23 (+)	0·09	0·20	0·09
Folic acid, $\mu g\ ml^{-1}$	0·01	0·058	0·008	0·040	0·02
Pyridoxine, $\mu g\ ml^{-1}$	0·043 (+)	0·046	0·02	0·059	0·042
Nicotinic acid, $\mu g\ ml^{-1}$	0·17 (+)	1·04 (+)	0·39	1·23	0·61
Cyanocobalamin, $ng\ ml^{-1}$	0·35	0·65	0·49	0·39	0·46

(+), Requirement for growth.
Values were calculated from figures published by Deguchi *et al.* (1985).
After Deguchi *et al.* (1985) and Scardovi (1986).

bifidum, *breve*, *infantis* and *longum* and, for comparative purposes, the vitamin requirements for growth.

Riboflavin was not synthesized by the strains studied (Deguchi *et al.* 1985), and the vitamin was reported by Scardovi (1986) to be a growth factor. The synthesis of ascorbic acid and biotin by *Bifid. longum* and *infantis* respectively was reported by Ballongue (1993), but no reference was quoted; the other organisms (*Bifid. breve*, *bifidum* and *adolescentis*) were also capable of producing these vitamins but at lower concentrations.

The production of such vitamins by bifidobacteria may improve the nutritional properties of the fermented milk product or the bioavailability of these vitamins in the human gut. The latter aspect merits further study.

BIFIDOBACTERIA IN THE HUMAN INTESTINAL TRACT

A vast literature exists on the intestinal microflora of human beings, and a diverse and complex ecosystem of bacterial populations is found. However, it is beyond the scope of this publication to review this topic in detail, and comprehensive data in this field have been published elsewhere (Savage, 1977*a*; Resnick & Levin, 1981*a*; Mitsuoka, 1982; Drasar & Barrow, 1985; Kim, 1988; Benno & Mitsuoka, 1986, 1991; Mutai & Tanaka, 1987; Berrada *et al.* 1989; Mitsuoka, 1989, 1990; Reuter, 1989; Reddy, 1990). At least 400 types of bacteria have been detected in the faeces of humans (Tannock, 1992; Lichtenstein & Goldin, 1993), and *Bifidobacterium* spp. are present in the colon of the intestinal tract. Finegold *et al.* (1974) have reported that *Bifid. adolescentis*, *infantis* and *longum* were among the 25 species of bacteria most frequently isolated from the faeces of Americans.

It is widely agreed (Mitsuoka, 1982; Stevenson *et al.* 1985) that the digestive tract of newly-born infants rapidly becomes colonized by bacteria as the result of oral contamination from the vagina during birth (Crociani *et al.* 1973; Mikelsaar & Mänder, 1993). Initially, a wide range of genera colonize the large intestine, and within 48 h of birth the stools of infants may contain up to $1{\cdot}0 \times 10^{10}$ cfu g^{-1} (Moreau *et al.* 1986). In breast-fed infants, this mixed population becomes dominated over the next few days by species of *Bifidobacterium*, with *Bifid. bifidum* being one of the most frequently isolated species (Yuhara *et al.* 1983). However, this pattern is not universal, and both Rose (1984) and Lundequist *et al.* (1985) observed that the substitution of the adventitious population by bifidobacteria was not as rapid as some reports would suggest. Given the complexity of the situation, these apparent contrasts are not unexpected, and it is suggested that the observed differences could arise for a number of reasons.

Environment. It is likely that the standards of hygiene in the delivery area and/or method of birth, e.g. natural as against Caesarean section, can alter both the speed of colonization of the colon and the species composition of the microflora.

Species identification. As mentioned earlier, the morphological and biochemical variations displayed by the bifidobacteria can make identification to species level a difficult operation, and it is possible that some confusion has arisen from this source.

Ecology of the bifidobacteria. Although the majority of bifidobacteria occupy the lumen of the colon, the walls of the colon provide a special ecological niche in which members of the genus can proliferate at the expense of other groups. For example, strains of *Bifid. bifidum* and *infantis* are capable of metabolizing the oligosaccharides found in the mucilage secreted by the wall cells of the colon, and hence the mucin tends to have a selective influence on the microflora (Croucher *et al.* 1983; Hoskins *et al.* 1985).

With respect to the actual species involved, the polysaccharides secreted by some strains of, for example, *Bifid. infantis* can initiate adhesion to the epithelial cells of the intestine, as can the lipoteichoic acids associated with the walls of Gram-positive bacteria (Courtney *et al.* 1981; Sato *et al.* 1982; Fischer, 1987; Fischer *et al.* 1987). Binding of the lipoteichoic acids of bifidobacteria to human epithelial cells appears, *in vitro*, to depend upon both cell concentration and the length of contact time, and hence there must always be a risk that cells of any given species will be voided from the body prior to adhesion, a point that confirms the general thesis that the frequency of species in stools need not reflect the population balance on the intestinal walls.

The most likely sites for interaction between the epithelial cells and bifidobacteria are proteins or glycoproteins capable of binding to the fatty acid fractions of the lipoteichoic acids (Op den Camp *et al.* 1985*b*), and Savage (1984) and Op den Camp *et al.* (1985*a*) suggest that the higher the level of fatty acids, the better the adhesion. In some cases, it has been suggested that a membrane from the host cell may grow around the bacterial cell – wholly or in part – to substantiate further the host–bacterium relationship (Guerina & Neutra, 1984). However, the significance of this possible interaction would seem to merit further study. It is important also that the ability of a host cell to accept a bacterial cell depends on both the age of the individual cell and the overall age of the human in question, an effect that could explain why the colonic microflora of children is observed to change with age.

The impact of these natural factors on the composition of the intestinal microflora may, of course, vary from host to host, and it is likely that this variation is magnified when comparisons are made between national groups.

Feeding regimen. A number of workers have confirmed the general trend that the colons of breast-fed infants have higher levels of bifidobacteria than those of bottle-fed neonates (Mitsuoka, 1982; Yuhara *et al.* 1983; Moreau *et al.* 1986). Whether or not the species frequency changes with type of feed is not clear, for while some workers have found *Bifid. bifidum* to be dominant following breast feeding, Biavati *et al.* (1984) isolated mixed populations, namely *Bifid. bifidum*, *infantis*, *longum* and *breve*, irrespective of the type of milk. In contrast again, Beerens *et al.* (1980) found that *Bifid. longum* was the most frequently isolated species from the stools of formula-fed infants, whilst Ballongue (1993) suggested that *Bifid. adolescentis* was likely to become the dominant organism following bottle feeding.

It has been suggested that the absence of specific stimulatory factor(s) in commercial feeds could be one factor involved in the apparent differences between the resultant microfloras. However, it is known that the precise composition of

human milk varies from mother to mother and between national groups. Such differences might further complicate the picture.

Age. At 1 month old, the colonic microflora of a bottle-fed infant may be dominated by bifidobacteria to the extent of 80% or more, but this figure gradually declines with age. In adults, bacteroides become the major group with bifidobacteria such as *Bifid. adolescentis* and *longum* forming, in numerical terms, the next most important population. *Bifid. bifidum* is another frequent inhabitant of the adult colon. However, the relative abundance of the bifidobacteria *vis-à-vis* 'putrefactive' genera such as *Escherichia* can have implications with respect to the healthy functioning of the colon, for there is considerable evidence that the bifidobacteria have a number of vital roles.

Natural functions of the bifidobacteria

At one time, it was assumed that the proximal end of the colon was dominated by Gram-positive bacteria including bifidobacteria, while the distal end contained a predominantly 'putrefactive' flora of less desirable species. More recently, Croucher *et al.* (1983) have suggested that the populations are much more dynamic, and hence that the undesirable flora may be much more restricted. Anything that favours this restriction could be of benefit to the host, and the bifidobacteria operate in this context in a number of ways.

The dominance of bifidobacteria at the walls of the colon provides competition for space and nutrients (Hill *et al.* 1986) at the expense of less desirable genera.
The bifidobacteria secrete lactic acid and acetic acids which may show both specific toxicity against certain species of yeasts or bacteria (Robinson & Samona, 1992) or, by lowering the pH of the intestinal contents, inhibit the growth of putrefactive organisms in general (Savage, 1977*b*).
The presence of bifidobacteria on the walls of the colon may induce cell-mediated immunity against *Esch. coli*, for example (Yamazaki *et al.* 1982), so preventing penetration of the epithelial layer by enteric bacteria, a protection that may be boosted by the bifidobacteria acting as stimulants of the immune system of the host.
Catteau *et al.* (1971) showed that bifidobacteria could deconjugate bile salts to free forms with more biocidal activity against adventitious bacteria.
Volatile fatty acids secreted by the bifidobacteria can act as peristaltic stimulants (Faure *et al.* 1982, 1984; Okamura *et al.* 1986), and this activity, together with control over the abundance of mucin at the intestinal surface, further assists in the healthy functioning of the colon.
A number of intestinal bacteria are able to synthesize nitrosamines, a family of compounds that are potentially carcinogenic. The degradation of these compounds by the bifidobacteria could play a role in reducing the risk of cancer of the colon. Whether or not this action is related to the alleged antitumour activities of bifidobacteria is not clear (Takano *et al.* 1985), but it remains a distinct possibility that a high population level of bifidobacteria in the colon can provide some protection against certain forms of tumour.
The bifidobacteria can metabolize ammonium ions, and their removal from the faeces may influence the movement of ammonia from the blood stream into the colon (Scardovi, 1986), a point of potential importance for patients with cirrhosis of the liver (Kurmann &Rašić, 1991).

The available evidence suggests, therefore, that a high level of bifidobacteria in

the colon is a desirable feature, and clinical studies on patients with this microflora depleted by antibiotic treatment, chemotherapy or radiation therapy have tended to confirm the importance of the above activities (Kageyama *et al.* 1984; Hotta *et al.* 1987; Mutai & Tanaka, 1987; Alm, 1991). It is against this background that the idea of dietary supplementation with dairy products containing bifidobacteria has gained credibility.

Therapeutic and prophylactic activities of bifidobacteria

Assuming that it is desirable that the bifidobacteria should be one of the major groups represented in the colon, then the possible routes for encouraging this situation are (i) feeding the subject with a diet that will stimulate his or her native microflora, (ii) consumption of dairy products manufactured with cultures of bifidobacteria (Fernandes & Shahani, 1989, 1990; Hitchins & McDonough, 1989) and (iii) the use of a suppository containing viable cells of bifidobacteria. The last approach may not be pleasant, but is probably just as effective. However, no results have been reported on using this method of introducing bifidobacteria into the human gut.

Although it is widely agreed that the colonic flora of infants can be influenced by the type of milk being used (breast or bottle), the reaction of 'normal' adults to dietary changes is still the subject of some controversy. Thus, whilst some authors believe that the microflora of a 'healthy' adult is essentially stable (Stark & Lee, 1982), others have found that foods can reduce (Mitsuoka, 1984) or stimulate (Roberfroid, 1993) the populations of bifidobacteria. Certainly, in some subjects, specific ingredients in a diet such as alcohol or garlic have been shown to have an adverse effect on the lactobacilli present at the distal end of the small intestine (Sellars, 1991), and it could be that similar materials have an impact on the microflora of the large intestine as well. The potential relevance of individual and/or national differences provides an additional complication, but the overall impression remains that changes in diet are likely to be important only in extreme situations.

However, the position with foods containing bifidobacteria may be different, and in assessing the potential advantages of consuming 'bioactive' milks or yogurts, it has to be assumed that:

consumption will be on a regular basis, and at a level of some 400–500 g product/week,
the dairy product will contain a minimum of $1{\cdot}0 \times 10^6$ cfu g^{-1} of product (Robinson, 1989) at the time of consumption and
the species of *Bifidobacterium* employed in manufacture will be of human origin, and able to withstand transit through the upper regions of the intestinal tract.

Given that these conditions can be met, then there is clinical evidence to support the thesis that the ingested bacteria may (i) stimulate the existing population of bifidobacteria, (ii) act in conjunction with the native flora or (iii) replace a microflora that has been seriously depleted by infection and/or antibiotics (Hotta *et al.* 1987; Mufai & Tanaka, 1987; Kurmann & Rašić, 1991).

The potential benefits for 'healthy' subjects is more difficult to predict, for it has to be assumed that such people will have a thriving healthy colonic microflora already. Nevertheless, adverse dietary changes could lead to a depletion of the natural flora, and it is of note that Kaloud & Stögmann (1969) demonstrated that the regular intake of *Bifid. bifidum* by 'healthy' infants did provide a degree of

protection against enteric infections. The reality of this potential benefit is strongly supported by the observations of Alm (1991).

In spite of this lack of direct evidence, it is worth recording that retail sales of bio-milks and bio-yogurts are growing rapidly both in Europe and North America. In the absence of any published surveys, it is not possible to predict the extent to which expected health benefits have contributed to this development. but it would seem unlikely that consumers would have acquired this intense product(s) loyalty without a genuine belief in its therapeutic and/or prophylactic properties.

TECHNOLOGY OF MANUFACTURE

The technology and biochemistry of fermentation, including the physicochemical changes that may occur in milk during the manufacture of yogurt and other fermented milk products, have been extensively investigated and reviewed by many research groups (Rašić & Kurmann, 1978, 1983; Tamime & Robinson, 1985, 1988; Rašić, 1986; Romond & Romond, 1990). Periodically the International Dairy Federation publishes monographs updating the technological and scientific aspects in the field of dairying and International Dairy Federation (1987, 1992*a*, *b*) review recent technological developments for fermented milks, hygienic design of dairy processing equipment and hygiene management in dairy plants.

However, the principal stages of production of any type of fermented milk (i.e. standardization of fat content, fortification of the milk solids. homogenization, de-aeration (optional) and high heat treatment) have much in common. Thus, the technical aspects, which will be considered in detail, include (i) the organisms that constitute the starter culture, (ii) temperature and period of incubation, (iii) the inoculation rate and (iv) production of bulk starter. Over the years equipment manufacturers have specifically developed and designed processes to meet the technological requirements of fermented milk products; the processing plants are more or less universal. For example, the use of de-aeration equipment in a yogurt production line may not be necessary, but it is highly desirable to de-aerate milk required for fermentation by bifidobacteria. The presence of oxygen in the milk may prolong the incubation period.

Over the past decade there has been a tremendous increase in the markets of Europe, North America and many other countries for dairy products (fermented or sweet) containing *Bifidobacterium* spp. originating from humans (Kurmann, 1983; Laroia & Martin, 1990; Hughes & Hoover, 1991; O'Sullivan *et al.* 1992; Hawkins, 1993). Such fermented milk products are made using a single genus, or in combination with other lactic acid bacteria as mixed starter cultures, and some examples follow.

Fermented products

A list of some current fermented milk products containing *Bifidobacterium* spp. available in different markets is shown in Table 8. It is evident that *Bifid. bifidum* and *longum* are widely employed as mixed starter cultures in combination with *Lb. acidophilus* and yogurt organisms (*Lb. delbrueckii* subsp. *bulgaricus* and/or *Streptococcus salivarius* subsp. *thermophilus* (*Str. thermophilus*)). In some instances, *Lactococcus* spp. and *Pediococcus acidilactici* are also used. Some of the products shown in Table 8 are also marketed in The Netherlands (Driessen & de Boer, 1989), as are different fermented milks made with *Lb. acidophilus* and *Bifid. bifidum* such as Biocultura® (stirred and drinking type) and Bio-Natuur® (set and stirred). The manufacture of some of these products is described below.

Table 8. *Fermented milks containing bifidobacteria currently available in different markets*

Product or trade name	Country of origin	Microflora present	Comments
AB milk products	Denmark	*Lactobacillus acidophilus* *Bifid. bifidum*	Developed by Chr. Hansen's Laboratory
Acidophilus bifidus yogurt	Germany	*Lb. delbrueckii* subsp. *bulgaricus* *Streptococcus thermophilus* *Lb. acidophilus* *Bifid. bifidum* or *longum*	Developed in 1969 and now produced in many countries
BA®	France	*Bifid. longum* Yogurt culture	Product is known as 'Bifidus Active'
Bifidus milk	Germany	*Bifid. bifidum* or *longum*	Developed in 1948 as baby food
Bifidus milk with yogurt flavour	UK	*Bifid. bifidum*, *longum* or *infantis*	Refer to text
Bifidus yogurt	Many countries	*Bifid. bifidum* or *longum* Yogurt culture	Refer to text
Bifighurt®	Germany	*Bifid. longum* CKL 1969 or DSM 2054 *Str. thermophilus*	Production method is similar to Bifidus yogurt; DSM 2054 is a slime-forming organism
Bifilakt® or Bifilact®	USSR	*Lactobacillus* spp. *Bifidobacterium* spp.	Refer to text
Biobest®	Germany	Similar to Bifidus yogurt Bifidobacteria type BAT	Developed by Laboratorium Visby and the product contains 'biogerm' grain
Biogarde®	Germany	*Lb. acidophilus* *Bifid. bifidum* *Str. thermophilus*	Refer to text
Bioghurt®	Germany	Similar to Biogarde®	Culture does not contain bifidobacteria
Biokys®	Czechoslovakia	*Bifid. bifidum* *Lb. acidophilus* *Pediococcus acidilactici*	Similar to the pharmaceutical product Femilact®
Biomild®	Germany	*Lb. acidophilus* *Bifidobacterium* spp.	Product is low in fat and without stabilizers or fruit flavours
Cultura®	Denmark	*Lb. acidophilus* *Bifid. bifidum*	Related to AB product
Diphilus milk®	France	As above	As above
Mil-Mil®	Japan	*Bifid. bifidum* *Bifid. breve* *Lb. acidophilus*	The product is sweetened with glucose and/or fructose and coloured with carrot juice
Ofilus®	France	*Str. thermophilus* *Lb. acidophilus* *Bifid. bifidum* or *Lactococcus lactis* subsp. *cremoris* *Lb. acidophilus* *Bifid. bifidum*	Contain 36 or 100 g fat kg^{-1} respectively
Progurt®	Chile	*Lc. lactis* biovar *diacetilactis* *Lc. lactis* subsp. *cremoris* *Lb. acidophilus* *Bifid. bifidum*	Refer to text
Sweet acidophilus bifidus milk	Japan	*Lb. acidophilus* *Bifid. longum*	Milk is not fermented after addition of starter culture
Sweet Bifidus milk	Japan Germany	*Bifidobacterium* spp.	As above.

After Schuler-Malyoth *et al.* (1968), Marshall *et al.* (1982), Gurr *et al.* (1984), Hansen (1985, 1989) and Kurmann *et al.* (1992).

Acidophilus bifidus yogurt. This is a fermented cows' milk product which is manufactured in many countries and is similar to Bifidus yogurt. The homogenized and heated milk (85 °C for 30 min or 90 °C for 5 min) is cooled to 40–42 °C, inoculated with separate cultures (yogurt, *Lb. acidophilus* and *Bifid. bifidum* or *longum*), packaged, incubated at 40–42 °C for 3–5 h, followed by cooling and storage (Rašić & Kurmann, 1978).

The therapeutic organisms should be of human origin, and the viable cell count in the fresh product averages $1–3 \times 10^7$ cfu ml^{-1} for each of *Lb. acidophilus* and bifidobacteria; the counts for the yogurt organisms are also high. Incidentally, the viable counts are influenced by (i) rate of inoculation, (ii) pH tolerance of the therapeutic strains and (iii) the manufacturing stages.

Bifidus milk. Standardized and fortified cows' milk (150–200 g total solids kg^{-1}) is homogenized and heated to 80–120 °C for 15 min. The milk is cooled to 37 °C, inoculated with starter culture (100 g l^{-1}; *Bifid. bifidum* or *longum*), and at pH ~ 4·5 the coagulum is cooled, packaged and moved to the cold store (Schuler-Mályoth *et al.* 1968; Rašić & Kurmann. 1983; Kurmann *et al.* 1992). Bifidus milk has the following characteristics: (i) it has a mild, acidic and slightly spicy taste, (ii) the molar ratio of lactic acid to acetic acid is 2:3, (iii) set or stirred types can be produced with or without added fruit flavour(s) and (iv) the viable count of *Bifid. bifidum* is $10^8–10^9$ cfu ml^{-1} with a decline of two log cycles during the storage period. Personal communications from starter culture suppliers and yogurt manufacturers in the UK indicate that fermented milks containing bifidobacteria are becoming more popular because they are milder in taste when compared with yogurt.

To improve the rate of acid development by the starter organism, the bulk starter is prepared in sterilized skimmed milk fortified with yeast extract, pepsin hydrolysed milk, corn extract or whey protein. The selection of more acid-tolerant strains is highly recommended (Klaver *et al.* 1993). Alternatively, concentrated starter cultures for direct-to-vat inoculation (DVI) can be used to avoid daily subculturing in the laboratory.

A similar product was developed in the UK using single strains of *Bifid. bifidum* 2203, *longum* 2259, *infantis* 2205 or *adolescentis* 2204 obtained from the National Collection of Food Bacteria (NCFB), formerly known as National Collection of Dairy Organisms (NCDO) (Marshall *et al.* 1982). Skimmed milk was fortified with ultrafiltered (UF) Cheddar cheese whey (concentrated 8-fold) and UF skimmed milk (concentrated 2-fold). The mixture (e.g. (g kg^{-1}) total solids 150, lactose ~ 50, protein 73, fat 130) was heated to 80 °C for 30 min, cooled to 37 °C, fortified with threonine (1 g kg^{-1}) and inoculated with starter culture (20 g l^{-1}). Incubation was at 37 °C for 24 h, after which the fermented milk was stored at 4 °C for 20 h before being assessed. The pH values after storage for 21 d ranged between 4·3 and 4·5, and the products made with *Bifid. bifidum, infantis* and *longum* had coagula similar to yogurt. The one made with *Bifid. adolescentis* was unacceptable as the pH was 5·1. The fermented milks had a 'walnutty' flavour and pleasant acidity, and the acetaldehyde content was 20–35 $\mu g\ g^{-1}$. The initial viable counts of all the bifidobacteria were 10^9 cfu g^{-1}, but a drop was observed after the storage period (to $< 10^5$ *Bifid. infantis*, 10^6 for *Bifid. longum*. 10^7 for *Bifid. bifidum* and 10^8 for *Bifid. adolescentis*).

A commercial method for the production of bifidus milk in India was reported by Misra & Kuila (1992). Skimmed ((g l^{-1}) fat 5, solids-not-fat 87) and other types of milks were heated to 90 °C for 30 min, cooled to 37 °C, inoculated with *Bifid. bifidum* NDRI (100 g l^{-1}, obtained from National Dairy Research Institute in India), and

incubated for 19 h. The products had a good storage life (i.e. a minimum of 3 weeks), good taste and a microbial population of 10^8 cfu g^{-1}. The antibacterial activity of the fermented milks against four pathogenic organisms was also studied, and it was dependent on (i) type of milk used (skimmed, cow, buffalo or reconstituted baby formula), (ii) degree of heating of milk, (iii) level of inoculum and (iv) concentration of sugar.

Bifidus and/or acidophilus drinks are becoming popular in certain markets, for example in the USA. The culture(s) (Viable®, supplied by Sanofi Bio-Industries, Waukesha, WI 53187-1609, USA) is thawed at 28–32 °C and added to cold pasteurized milk, followed by packaging. The viable cell count is $> 2 \times 10^6$ cfu ml^{-1} at the end of a 2 week shelf life, which complies with the statutory regulations in California and Oregon (Anon. 1993*a*). A similar product containing *Lb. acidophilus* and *Bifid. bifidum* is also marketed in the USA by using a mixed starter culture called Nu-trish A/B® which is supplied by Chr. Hansen's Laboratories in the USA (Hoover & Hughes, 1991). Thus these products are sweet in taste and not fermented, but they offer a way to provide an abundance of bifidobacteria to the consumer.

Bifidus yogurt. A mixed starter culture (*Bifid. bifidum* or *longum*, yogurt culture with or without *Lb. acidophilus*) is used at a rate of 50–100 g l^{-1} to ferment milk at 40–42 °C for 3–5 h (Rašić, 1986; Kurmann *et al.* 1992). Normally, a DVI bifidus culture is used plus *Str. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* at a level of 1–10 g l^{-1} for simultaneous fermentation. Alternatively, the different starter cultures are used to ferment the milks separately, followed by mixing before cooling and packaging.

Bifighurt®. This fermented milk is similar in production to Bifidus milk or Bifidus yogurt, but the starter culture consists exclusively of *Bifid. longum* CKL 1969 or DSM 2054 (a slime-producer) and *Str. thermophilus*. The bifidobacterial count in the product is 10^7 cfu ml^{-1}, and practically only L(+) lactic acid (95%) is produced.

Bifilakt® or Bifilact®. This is an antacidic milk product developed for the treatment of gastrointestinal diseases in children. A mixture of lactose (30 g) and maize starch (30 g) is dispersed in a small volume of water and added to every litre of boiled whole milk. The mixture is then sterilized, fortified by the addition of lysozyme (0·05 g l^{-1}) in physiological saline, inoculated with 50 ml l^{-1} of each starter culture consisting of *Bifidobacterium* spp. and *Lactobacillus* spp., packaged in sterile containers and incubated at 37 °C for 18–20 h (Dorofeichuk *et al.* 1983). The finished product is sweet in taste (i.e. pH 5·8–6·0), stable for a week at < 10 °C and the cell count is 10^8–10^9 cfu ml^{-1}. The protein and fat contents of Bifilact® are 42·3 and 37 g kg^{-1} respectively.

Biobest®. This product, a low-fat fermented milk which is produced in Germany by using a mixed culture containing bifidobacteria type BAT (Laboratorium Visby, Tønder, Denmark), is similar to Bifidus yogurt but with the addition of 'biogerm' grains and fruits. The yogurt culture is used at a very low inoculation rate (0·1–1 g l^{-1}) (Rašić & Kurmann, 1983).

Biogarde®. This fermented milk product was developed in Germany by Schuler-Malyoth *et al.* (1968), and at present it is manufactured in many countries under licence. The standardized milk is homogenized, heated to 90 °C for 10 min or 95 °C for 5 min, cooled and inoculated with mixed bulk starter culture (60 g l^{-1}) which contains *Lb. acidophilus* (human origin), *Bifid. bifidum* (human origin) and *Str. thermophilus*. The last organism is incorporated for fast acid development (Klupsch, 1983, 1984; Rašić & Kurmann, 1983). The mixture is packaged, incubated for 3·5 h at 42 °C until coagulation, and then cooled.

When this product was originally launched in the 1960s, the bulk starter cultures were produced as monocultures, and the rate of inoculum required was 100–200 g l^{-1} in order to ensure sufficient bifidobacteria (i.e. 10^6–10^8 cfu ml^{-1}) in the milk and, consequently, slightly higher counts in the end product (Tamime & Robinson, 1988). At present, the bulk starter culture is prepared as follows. Biogarde® culture nutrient medium (~ 15 g l^{-1}) is dissolved in water, mixed with bulk starter milk, heated to 90 °C for 10 min, cooled to 42 °C and inoculated with a laboratory grown liquid culture or DVI culture. The latter system is more widely practised in order to ensure a shorter production time and high numbers of viable cells (Tamime & Robinson, 1988). The milk is then incubated for a period of 4·5–6·5 h, cooled and stored at 8 °C (Kurmann *et al.* 1992).

Set or stirred type fermented milks are produced which could be flavoured with fruits or other flavourings. The natural flavour Biogarde® has a mildly acidic taste, and contains mainly L(+) lactic acid (85–90%) and about 10^7–10^8 and 10^6–10^7 cfu ml^{-1} of *Lb. acidophilus* and *Bifid. bifidum* respectively, in addition to an abundant count of *Str. thermophilus*. The total production figure of Biogarde® was 7000 tonnes in 1986 (Tamime & Robinson, 1988), but no figures are available for the present date.

It is worthwhile pointing out that Bioghurt® is a product closely related to Biogarde®, but the bifidobacteria are not included with *Lb. acidophilus* and *Str. thermophilus* and/or a yogurt culture. However, formerly *Lc. lactis* subsp. *lactis* biovar *taette* was used instead of the streptococci (Kurmann *et al.* 1992). Similarly, the Swiss sour milk (Bio-yogurt) is a synonym of a product made from organically produced milk, and only a yogurt starter culture is used, i.e. one containing no *Lb. acidophilus* or bifidobacteria.

Biokys®. This is a Czechoslovakian fermented cows' milk ((g kg^{-1}) total solids 150, fat 35) which has therapeutic properties similar to the pharmaceutical product Femilact®. The milk is homogenized, heat treated, cooled to 30–31 °C and inoculated with mixed starter culture (20–50 g l^{-1}, *Bifid. bifidum*, *Lb. acidophilus* and *Ped. acidilactici* in a ratio of 1:0·1:1) (Hylmar, 1978). At the desired acidity, the coagulum is stirred, cooled and packaged. However, in a study of a similar starter combination with a ratio of 45:10:45, growth activity at 37 °C was more than at 30 °C (Doležálek & Plocková, 1981). At present, Biokys® is produced with the following mixed cultures: cream DL starter, *Lc. lactis* subsp. *cremoris* (slime producing strain), *Bifid. longum*, *Bifid. bifidum* and/or *Lb. acidophilus* (Hušek *et al.* 1990).

Biomild®. This product is a German fermented milk made with *Lb. acidophilus* and bifidobacteria cultures, 'natural' in flavour and sold in 500 g packs.

Cultura AB®. There is a wide range of lactic starter cultures for the manufacture of fermented milks, buttermilk, AB-yogurt and so on. These cultures were developed by Chr. Hansen's Laboratory in Denmark (Rašić & Kurmann, 1983; Tamime & Robinson, 1988). Protein-enriched whole milk is homogenized, heat treated, cooled to 37 °C, inoculated with DVI frozen concentrated culture (or bulk starter), and incubated for 16 h followed by cooling.

The set-type product is normally produced in 150–500 ml containers. The shelf life is ~ 20 d. and the viable cell count when fresh is $\geqslant 10^8$ cfu ml^{-1} for both *Lb. acidophilus* and *Bifid. bifidum* (Kurmann *et al.* 1992).

Ofilus®. This French fermented milk (set type) made from cows' milk was launched in 1987. Two types of Ofilus® products are available, Ofilus® 'nature' (36 g fat kg^{-1}) made with mixed starter cultures consisting of *Str. thermophilus*, *Lb. acidophilus* and *Bifid. bifidum* and Ofilus® 'double douceur' (100 g fat kg^{-1}), which is

milder in taste because the lactic *Streptococcus* is replaced with *Lc. lactis* subsp. *cremoris* (Kurmann *et al.* 1992). Clinical studies with Ofilus®, and including other dairy products containing *Lb. acidophilus*, *Bifid. bifidum* and/or mesophilic lactococci, were carried out by Marteau *et al.* (1990). They observed that regular consumption of fermented milk products containing viable cells (e.g. *Bifid. bifidum* strain B1, 10^8 cfu g^{-1}) increases the activity of β-glucosidase (EC 3.2.1.21), which is implicated in the colonic fermentation of cellulose. However, further studies have been suggested by the same authors to determine the mechanics of these modifications and the fate of the organisms in the intestinal tract.

Progurt®. This Chilean protein-enriched product ((g kg^{-1}) protein 62, lactose ~ 30, fat > 50, ash 7) is made from cultured skimmed milk, and mixed with buttermilk (< 500 g kg^{-1}) and cream (Schacht & Syrazynski, 1975). The skimmed milk is heated to 95 °C for a few seconds, cooled and inoculated with mesophilic starter cultures (10–30 g kg^{-1}, *Lc. lactis* subsp. *lactis* biovar *diacetilactis* and *Lc. lactis* subsp. *cremoris* at a ratio of 1:1). After 12–18 h, the acidity reaches 8–9 g lactic acid l^{-1}, and the gel is heated to < 45 °C to separate the whey (a process similar to the traditional method for the manufacture of ymer (see Tamime & Robinson, 1988). The partly concentrated coagulum is standardized with cream, homogenized, cooled and packaged. For the manufacture of therapeutic Progurt®, *Lb. acidophilus* and/or *Bifid. bifidum* are added at 5–10 g l^{-1} to the concentrated and fermented skimmed milk before the homogenization stage. This product should not be classified as strained yogurt (see section below) because the total solids content is < 220 g kg^{-1}.

It is evident from consideration of the above products that a wide range of mixed starter cultures is used in order to achieve high cell counts of bifidobacteria at the end of shelf life. In the 1980s, Reuter (1990) conducted a survey of fermented milk products containing bifidobacteria in Germany, Japan and France. He found that *Bifid. longum* is widely used in Germany, and the higher the addition rate of yogurt and *Lb. acidophilus* starter cultures, the greater the variation of the bifidobacterial counts. The most suitable combination is *Bifid. longum* with *Str. thermophilus*, but the manufacturing procedure is very laborious. The recent trend towards products containing high counts of bifidobacteria could possibly be due to (i) selection of strains that are more acid-tolerant, for example, the substitution of *Bifid. longum* with *Bifid. animalis* (this organism is not of human origin) and (ii) improvement in the technology of production to provide better anaerobic conditions. However, *Bifid. longum*, *breve* and *infantis* strains are found to tolerate oxygen better than the sensitive *Bifid. adolescentis* (Shimamura *et al.* 1992). He also found that Japanese fermented milks contained sufficient viable counts of *Bifid. longum* (2×10^7 cfu g^{-1} when fresh and 4×10^5 cfu g^{-1} after 18 d storage), while the viability of *Bifid. breve* and *bifidum* only decreased by 1·5 and 0·5 log cycles in 20 d. Lastly, he found that the numbers of bifidobacteria in French products averaged $1{\cdot}7 \times 10^7$ cfu g^{-1}, and the main species were *Bifid. longum* and *animalis*; however, *Bifid. animalis* may not survive in the human intestine. Recently, a study by a French scientist reported that one of the two bifidobacterial strains isolated from commercial bifidus milk survived well ($\geq 10^7$ cfu g^{-1}) in an *in vitro* simulated gastric transit, while a second strain was much less resistant (Berrada *et al.* 1991).

In a recent survey of yogurt products containing bifidobacteria (Dibb *et al.* 1991; Anon. 1993*b*) the viable cell counts of these organisms ranged from 1×10^5 to 3×10^7 cfu g^{-1}. These differences could be mainly attributed to the selection of commercial strains that can have higher survival rates in acidic products (Sakai *et al.* 1987; Robinson, 1990; Misra & Kuila, 1991*a*; Martin & Chou, 1992; Anon. 1993*a*).

Strained or Greek-style yogurt

Concentrated yogurt is a fermented milk product manufactured from full fat and unsweetened 'natural' yogurt by removing some of the whey. The product is manufactured in many countries and is known as *labneh*, *lebneh* or *labnieh* (in the eastern Mediterranean countries), *laban zeer* (in Egypt and Sudan), *tan* or *than* (in Armenia), *stragisto* or *sakoulas* (in Greece), *kis*, *kurut*, *torba* or *tulum* (in Turkey), *mastou* or *mast* (in Iraq and Iran), *basa*, *zimme*, *kiselo* or *mleko-slano* (in the Balkan countries) and *Greek-style* (in the UK) (Tamime & Crawford, 1984; Tamime & Robinson, 1988; Kurmann *et al.* 1992).

In the Lebanon and Saudi Arabia, the compositional standards are typically (g kg^{-1}) total solids 220–260, fat 90–100, salt (optional) 10. The processing methods are (i) traditional, using a cloth bag to drain the whey, (ii) quarg centrifugal separators suitable for fermented skimmed or whole milk, (iii) UF of milk prior to fermentation, (iv) UF of warm yogurt and (v) product formulation. Details of these processing methods, and in particular the UF of warm yogurt, have been reported by Tamime *et al.* (1989*a*, *b*. 1991*a*, *b*) and Tamime (1993).

The quality of Greek-style yogurt made by UF using cows', ewes' and goats' milk and fermenting the milk with *Bifid. bifidum* Bb-12 (concentrated and frozen DVI culture obtained from Chr. Hansen's Laboratory in Denmark) was reported by Mahdi (1990) and Mahdi *et al.* (1990). The viable cell count of the DVI starter culture was $2{\cdot}0–2{\cdot}3 \times 10^{11}$ cfu g^{-1}. and in the UF Greek-style product the bifidobacterial counts ranged between $4{\cdot}6 \times 10^{5}$ and $4{\cdot}1 \times 10^{7}$ cfu g^{-1} depending on the type of milk used. Predominantly the L(+) lactic acid isomer was produced by the starter culture, and the range of compositions of these products was (g kg^{-1}) total solids 215–226, fat 85–106, protein 73–76. ash 7–9; the pH ranged between 4·25 and 4·54. The organoleptic scores given by the taste panellists to the bifidus products (i.e. flavour and aroma) were low compared with similar products made with *Lb. acidophilus* or a yogurt starter culture. because of the appreciable presence of acetic acid in the product.

Frozen cultured ice-creams

Over the past decade. these products became popular among consumers simply for the therapeutic properties of yogurt. The frozen products resemble ice-cream in their physical state and they have the sharp acidic taste of fermented milks combined with the coldness of ice-cream. Before freezing, the product is fortified with sugar and stabilizers and emulsifiers which are required to maintain the air-bubble structure during the freezing process. Some technological aspects have been reported by Rašić & Kurmann (1978), Tamime & Robinson (1985), Arbuckle (1986), Bielecka *et al.* (1988), Martinou-Voulasiki & Zerfiridis (1990), Williams (1990), Opdahl & Baer (1991) and Mashayekh & Brown (1992).

Biogarde® ice-cream was marketed in Germany in the mid 1980s (Anon. 1986), but no technological aspects were reported. According to Kurmann *et al.* (1992), Biogarde® ice-cream contained up to 10^{8} cfu g^{-1} viable cells of *Lb. acidophilus*, 10^{7} cfu g^{-1} of *Bifid. bifidum* and an abundance of *Str. thermophilus*. The same organisms have been used in laboratory studies in the USA for the manufacture of hard and soft serve ice-cream (Holcomb *et al.* 1991; Laroia & Martin, 1991*a*; Hekmat & McMahon, 1992). Different formulations and methods were used by these authors to prepare the frozen products, and Fig. 3 illustrates one such example. The survival rates of *Lb. acidophilus* and *Bifid. bifidum* after 17 weeks storage were very good, and

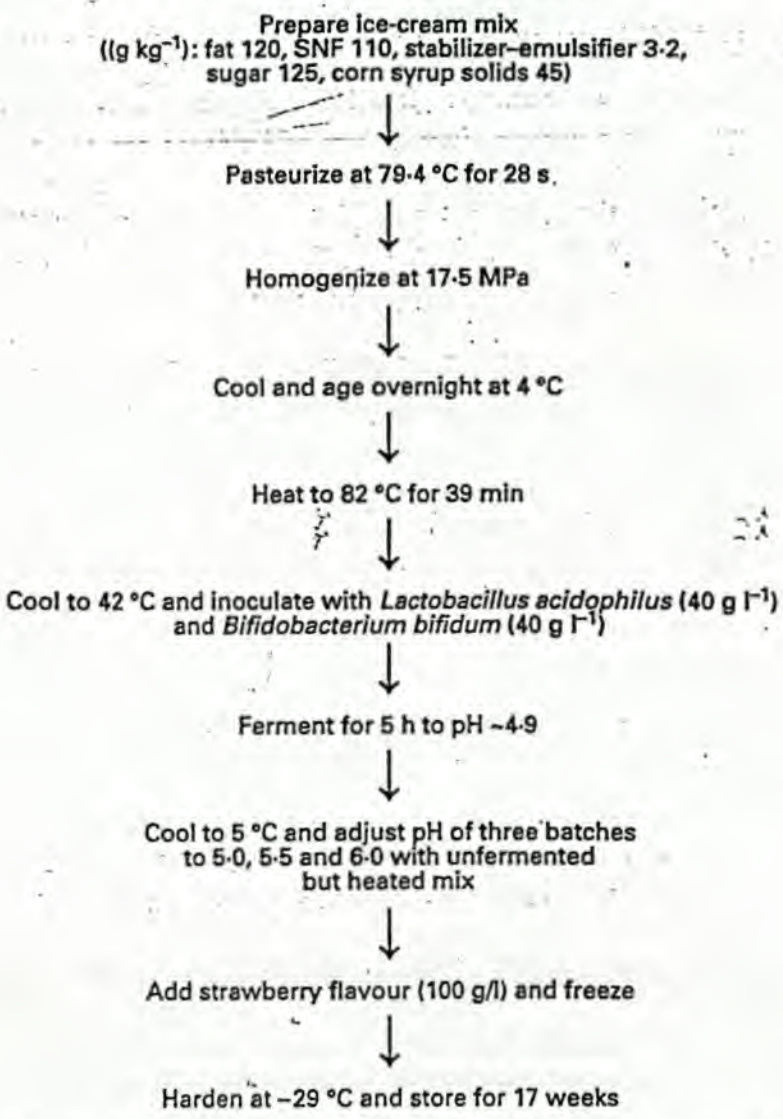

Fig. 3. Flow diagram of the manufacturing stages of frozen cultured ice-cream. (Adapted from Hekmat & McMahon, 1992.)

at pH 5·5 the highest viable cell counts were observed (4×10^6 and 1×10^7 cfu ml^{-1} respectively) which corresponded to a decrease of 2 and 1 log cycles respectively when compared with freshly frozen products (Hekmat & McMahon, 1992). Similar results were also reported by Laroia & Martin (1991*a*).

In soft serve ice-cream, the numbers of *Bifid. bifidum* were roughly the same before and after freezing ($1{\cdot}18 \times 10^8$ and $1{\cdot}23 \times 10^8$ cfu ml^{-1}) at pH 5·4–6·5, i.e. after a 6 h holding period at -5 °C which simulates the processing conditions of the frozen mix in the freezer compartment before discharge (Holcomb *et al.* 1991). For no apparent reason, the viable cell count of *Lb. acidophilus* under the same processing conditions increased. In the same study, both *Bifid. bifidum* and *Lb. acidophilus* were exposed to 10 mM-HCl for 2 h at 37 °C simulating the time required to pass through the stomach, and to fortification of the de Mann–Rogosa–Sharpe (MRS) medium with different concentrations of oxgall (1·5–4·5 g l^{-1}) to determine bile resistance. The acid conditions reduced the microbial count of *Bifid. bifidum* but not *Lb. acidophilus*, and the presence of oxgall in the plating medium did not cause any significant difference in the counts before or after freezing.

In an effort to increase the survival rate in frozen cultured ice-cream, a Canadian group evaluated two mechanisms. Firstly, they grew *Bifid. longum* in a demineralized and deproteinized whey medium to obtain high counts ($1{\cdot}7 \times 10^9$–$2{\cdot}5 \times 10^{11}$ cfu g^{-1} in a freeze-dried preparation) before addition to the ice-cream mix prior to freezing. Secondly, they mixed *Bifid. longum* ATCC 15707, *infantis* ATCC 15697 and *adolescentis* ATCC 15703 separately with two types of bifidogenic factors, neosugars and Jerusalem artichoke flour (Modler *et al.* 1990*b*; Modler & Villa-Garcia, 1993). In both studies the ice-cream was stored for ~ 11 weeks and the results could be summarized as follows. With the freeze-dried preparation of *Bifid. longum*, the highest count was obtained when using a strain from Japan and, at pH 5·8, a decline of only 1 log cycle in bacterial count was observed after 11 weeks storage. Encapsulating the bifidobacteria in milk fat prior to freezing did not improve the survival rate. At freezing, ~ 10% loss in viable counts occurred possibly due to the incorporation of air to achieve 100% overrun, and *Bifid. infantis* (ATCC 15697) was the least sensitive. The maximum decline in viable cell counts did not exceed 1 log cycle. The use of Jerusalem artichoke flour imparted a flavour that was unacceptable to the taste panellists.

Thus, it is evident from these limited trials that the choice of bifidobacterial strains (i.e. tolerant to acidic conditions and insensitive to oxygen), and the use of high bacterial counts prior to freezing can ensure high survival rates in the product.

Fresh cheese

Very limited data are reported in the literature regarding the introduction of bifidobacteria into cheeses. However, Biobest® culture (Table 8) has been used during the manufacture of Thermo Quarg (Klupsch, 1983; Rašić & Kurmann, 1983), and *Bifid. bifidum* in Cottage cheese (Anon. 1993*a*). These cultures were most likely added to the curd at the creaming stage before packaging.

Studies on the use of *Bifid. bifidum* in Tvaroh (a fresh cheese) were carried out in Czechoslovakia (Lang & Lang, 1978*a*, *b*). The bifidobacteria were used in combination with a cream starter culture, and the viable count of lactic acid bacteria in the fresh product was ~ 10^9 cfu g^{-1}, of which 35% was *Bifid. bifidum*. After 3 d the bifidobacteria count fell by 66% as compared with 83% for the lactococci, and the *Bifid. bifidum* became predominant in the product. The actual dessert is made with (g kg^{-1}) Tvaroh 200, whipped cream 500, sugar 150, pasteurized fruit pulp 150.

Bifidus milk powder

A wide range of these products is on the market, and they have been developed as formula feeds for infants. Although such products are not primarily considered 'pure' dairy products made by the industry, the dietary aspects take into account (i) the need to modify cows' milk to resemble the chemical composition of human milk, (ii) the addition of a 'bifidus' factor(s) to mimic that present naturally in milk (see section on growth factors) and (iii) the use of such products as vehicles for the implantation of *Bifidobacterium* spp. in infants who are bottle-fed.

Rašić & Kurmann (1983) reviewed some of these products which may contain *Bifid. bifidum* alone or in combination with *Lb. acidophilus* and *Ped. acidilactici*. A typical composition is (g kg^{-1}) fat 220, protein 133. carbohydrates 600, minerals 29 plus vitamins A, D and C and iron. A daily intake of 10^7 cfu g^{-1} of freeze-dried *Bifid. bifidum* in the diet of bottle-fed babies ensures the implantation of the organism in the gut after 2–3 d (Rašić & Kurmann, 1983). A similar Czechoslovakian product called Femilact® is manufactured as follows. Heat-treated cream (120 g fat kg^{-1}) is

fermented with *Bifid. bifidum*, *Lb. acidophilus* and *Ped. acidilactici* (at a ratio of 1:0·1:1) at 30 °C, cooled and mixed with heated vegetable oil, lactose, whey protein and vitamins. The mixture is homogenized and spray dried. The reconstituted baby milk contains lactic acid (2·5 g l^{-1}) and has a viable cell count of ~ 10^8–10^9 cfu ml^{-1} with a decline by 1 log cycle after storage for 2 months (Kurmann *et al.* 1992).

These products have been developed by industrial organizations, and the available technical information is somewhat limited. Nevertheless, in the 1970s *Bifid. bifidum* strains were grown satisfactorily in combination with *Lb. acidophilus* and *Lc. lactis* subsp. *lactis* biovar *taette* for the enrichment of dried infant foods. A reduction in the viable cell count of *Bifid. bifidum* was observed as the milk total solids reached 300 g kg^{-1} in the evaporator (Anon. 1974), but no exact count of bifidobacteria in the dried product was given. Recently, Nagawa *et al.* (1988) produced a bifidus milk powder using malt extract and skimmed milk and four different strains of *Bifid. longum*. Both the skimmed milk and malt extract were used in equal amounts in solution (200 g l^{-1}), sterilized at 140 °C for 3 s, cooled to 37 °C and inoculated with *Bifidobacterium* spp. (30 g l^{-1}). At pH 5, the viable cell count was 5×10^9 cfu ml^{-1}, and the fermented milk was frozen at −20 °C followed by lyophilization at 20 °C for 24 h. Adjustment of the fermented milk to pH 8–9 before freeze drying increased the survival rate after storage for 30 d at 35 °C by 20 times compared with non-adjusted milk. The viable cell count of *Bifid. longum* ATCC 15707 in the alkaline milk was > 10^9 cfu g^{-1} after the storage period.

Pharmaceutical products

Special dietary preparations containing viable cells of *Bifidobacterium* spp., often in combination with other therapeutic organisms, are available in different markets as freeze-dried tablets. The primary objective of these products is to achieve implantation of the bacteria in the gut during the treatment of various ailments and diseases. Some examples are gastrointestinal disorders, after antibiotic therapy, adjusting imbalances of the microflora in the gut, liver diseases, chronic constipation, chronic duodenitis and peptic ulcers in children, and after irradiation therapy.

Some examples of pharmaceutical products made from cows' milk and containing viable counts of *Bifid. bifidum* or other *Bifidobacterium* spp. are Bifider® (Japanese), Bifidogène® (French) and Liobif® (Yugoslavian). Two products developed in Germany and containing high numbers of *Bifidobacterium* spp. have the following compositions (g kg^{-1}). Eugalan Töpfer Forte® (milk protein 94, plant protein 7, lactose 626. lactulose 59 plus 30 minerals) is a fat-free and gluten-free preparation. Euga-Lein Töpfer contains fat 18, protein 151, carbohydrates 711, minerals 31, dietary fibre 48, vitamin C 2. Omniflora®, which is made in Germany, contains *Lb. acidophilus*, *Bifid. longum* and a saprophytic *Escherichia coli* (Kurmann *et al.* 1992).

Although it could be argued that the presence of such organisms in the human gut is beneficial to the body, the efficacy of any claims relating to dietary preparations merits proper medical investigation.

Miscellaneous applications

UF milk is used in the industry for the manufacture of certain cheese varieties where mesophilic and thermophilic lactic acid bacteria are employed (Tamime & Kirkegaard. 1991: Mistry & Maubois. 1993). The growth behaviour of *Bifid. bifidum* ATCC 15696 and *Bifid. longum* ATCC 15708 was studied by Ventling & Mistry (1993) in UF skimmed milk at different concentration factors between 2:1 and 5:1. Owing to the buffering capacity of UF milk, both cultures had counts of 10^8–10^9 cfu ml^{-1},

and the observed pH remained high (5·35–5·5). Growth of *Bifid. bifidum* in skimmed milk (with or without UF) modified the bifurcated Y shape, the microbial chain was shorter and the cells became oval in shape. The same authors recommended a number of potential developments of bifidobacteria in this area, which could include bulk starter and yogurt production from UF milk. However, the possible use of bifidobacteria in cheesemaking should not be overlooked.

The utilization of soya milk for the manufacture of closely related products provides a good source of protein in countries deficient in animal protein. Maslov *et al.* (1990) prepared a fermented soya product using *Bifidobacterium* spp. and, after 16 h incubation at 37 °C, the viable cell count ranged between 10^8 and 10^9 cfu ml^{-1} and the lactic acid reached ~ 50 g l^{-1}. In a separate study, Murti *et al.* (1993) evaluated the quality of yogurt using cows' and soya milk. The culture combinations used (yogurt strains with or without *Bifidobacterium* spp.) were isolated from commercial products in France. The results with soya yogurt were (i) the use of bifidobacteria with yogurt cultures suppressed slightly the growth of *Lb. delbrueckii* subsp. *bulgaricus*, (ii) the ethanol content and quantities of other volatile compounds were slightly higher in yogurts containing *Bifidobacterium* spp. and (iii) *n*-hexanal was absent in all the products.

Thus, it is possible to suggest that soya milk could be used successfully for the manufacture of a 'vegetarian' yogurt for the European and North American markets. From the above studies, it is safe to conclude that a mixed culture of *Str. thermophilus* and bifidobacteria could be an ideal combination.

ENUMERATION OF BIFIDOBACTERIA

The culture media that were originally devised for isolation and enumeration of *Bifidobacterium* spp. from the intestinal tract or faeces of humans will not be reviewed. However, some of these media have been slightly modified for the enumeration of bifidobacteria in fermented dairy products and pharmaceutical preparations when a single culture is used. The difficulty arises in enumeration when mixed cultures are used, such as *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, *Str. thermophilus* and *Ped. acidilactici* in combination with *Bifidobacterium* spp. In order to allow selective enumeration of bifidobacteria on an agar medium certain aspects have been considered, such as provision of special growth factors, lowering the redox potential, fortification of the medium with blood and addition of antimicrobial substances that can inhibit the growth of lactic acid bacteria but not bifidobacteria.

An extensive review of culture media for detection and enumeration of bifidobacteria in fermented milk products has recently been published by Rašić (1990), and some examples are shown in Table 9. It is safe to assume that the medium developed by Teraguchi *et al.* (1978) for the selective enumeration of bifidobacteria has received the greatest attention, but the paper is published in Japanese, and a translation has only recently appeared (Laroia & Martin, 1991*b*). The same authors (Laroia & Martin, 1991*a*) inadvertently omitted the blood component from the formulation, a fact observed by Modler & Villa-Garcia (1993); such information is important for researchers trying to prepare Teraguchi's medium in-house.

Acidified media such as MRS, Rogosa and others have been used successfully by some research groups in this field for the enumeration of bifidobacteria in mixed cultures (Robinson, 1990; Holcomb *et al.* 1991; Samona & Robinson, 1991), while others reported their limited selectivity (Wijsman *et al.* 1989; Kneifel & Pacher, 1993). Nevertheless, it is evident that further development in media formulation is

Table 9. *Examples of some selective media used for the enumeration of* Bifidobacterium *spp. from fermented dairy products*

Basal medium	Comment	References
Modified Columbian medium with the addition of (g l^{-1}): glucose 5, cysteine hydrochloride 5, agar 5, containing propionic acid (5 or 10 ml l^{-1}) and adjusted to pH 5.	Selective medium against many groups of organisms including *Lactobacillus* spp. and *Streptococcus* spp., and 19 bifidobacteria strains showed improved growth: the correlation was ±0·4 log units for all strains except one.	Beerens (1990)
Trypticase–phytone–yeast (TPY) medium with the addition of dicloxacillin (2 μg ml^{-1}): this medium was more suitable than Mann–Rogosa–Sharpe (MRS) agar to select bifidobacteria.	Growth of lactobacilli and streptococci was inhibited and human strains of *Bifidobacterium* spp. grew well; a study was carried out on pure strains and commercial products (e.g. Ofilus®, BA®, Bifidus yogurt, Fromage bifidus etc).	Sozzi *et al.* (1990)
Glucose–blood–liver (BL) agar with added neomycin–paromomycin–nalidixic acid–lithium chloride (NPNL).	It is considered to be the reference medium for selective enumeration of bifidobacteria found in fermented dairy products: it is time consuming to prepare and some ingredients are heat-sensitive and must be filter-sterilized.	Teraguchi *et al.* (1978)
Modified liver–cystine–lactose (LCL) agar (i.e. cystine was omitted and NaCl concentration was reduced to 2 g l^{-1}) with added lithium chloride (2 g l^{-1}) and sodium propionate (3 g l^{-1}): this formula is known as LP agar.	Selective enumeration of bifidobacteria from commercial samples of yogurt was effective; nine strains of bifidobacteria used in dairy products grew well with the exception of *Bifid. longum* B118; all strains of lactobacilli, yogurt cultures and lactococci were inhibited except one *Str. thermophilus* YS4 and one *Lactococcus lactis* subsp. *cremoris* Sc 15.	Lapierre *et al.* (1992)
Modified VF-Bouillion agar with added lithium chloride (0·5 mg l^{-1}), sodium lauryl sulphate (20 μg ml^{-1}), sodium propionate (5 mg ml^{-1}), and neomycin sulphate (10 μg ml^{-1}).	This medium is used with a triple-layer diffusion technique to selectively enumerate *Bifid. bifidum* and modified Briggs agar with streptomycin sulphate (1200 μg ml^{-1}) to enumerate *Lb. acidophilus* antibiotic-resistant strain: application is for the determination of viable cells of these organisms in probiotic dried products or after rehydration.	Calicchia *et al.* (1993)
Transgalactosylated oligosaccharide (TOS) agar medium supplemented with NPNL. Milieu Selectif *Bifidobacterium* spp. (MSB) agar or Arabinose agar (where L-arabinose is substituted for TOS).	All these media are selective for enumeration of bifidobacteria from dairy products: arabinose agar is only suitable for bifidobacteria strains capable of fermenting arabinose, e.g. *Bifid. longum* and *adolescentis* (see Table 3).	Wijsman *et al.* (1989)
Galactose agar with added lithium chloride (0·4 g l^{-1}) and galactose (10 g l^{-1}) as selective inhibitory agents.	Five bifidobacteria strains were isolated from eight commercial products made in Europe: only one strain was classified as *Bifid. longum* and the rest *Bifid. animalis*; the viable bifidobacteria counts ranged between 10^4 and 10^7 cfu ml^{-1}.	Iwana *et al.* (1993)
MRS agar with added neomycin sulphate (100 mg l^{-1}), nalidixic acid (15 mg l^{-1}) and lithium chloride (3 g l^{-1}) (NNL) solution.	This medium is selective for bifidobacteria and by using the oxygen-reducing membrane fraction (ORMF) the plates could be incubated aerobically as a substitute for anaerobic jars: no difference between the mean counts in log(cfu ml^{-1}) was observed where other growth conditions were compared.	Burford (1989)

Table 9. (*contd.*)

Basal medium	Comment	References
Rogosa medium with added 5-bromo-4-chloro-3-indol-β-D-glucopyranoside (40 μg ml^{-1}); known as X-Glu agar.	Non-selective for bifidobacteria and yogurt cultures, but selective for *Lb. acidophilus*, and is recommended for the enumeration of lactobacilli in fermented milk products.	Kneifel & Pacher (1993)
Reinforced clostridial agar (RCA) fortified with a wide range of different inhibitory components; the medium is called BIM-25.	This is a selective medium, for isolation of *Bifidobacterium* spp. of human origin from natural aquatic environments: however, its application for routine examination of fermented milk merits further investigation because the inhibitory agents would prevent the growth of yogurt and *Lb. acidophilus* cultures.	Muñoa & Pares (1988)
Modified Teraguchi *et al.* (1982) method also contains lithium chloride (3 mg l^{-1}); the medium is known as MGA.	Growth of *Lb. acidophilus* was inhibited but not *Lb. casei* subsp. *casei* when enumerated with mixed cultures of *Bifid. breve* and/or *infantis*.	Cheng & Nagasawa (1983)
Reinforced clostridial Prussian blue (RCPB) agar.	Yogurt cultures and *Bifid. bifidum* could be enumerated effectively by their distinct colony morphologies.	Onggo & Fleet (1993)
YN-6 with added nalidixic acid (80 μg ml^{-1}), neomycin sulphate (2·5 μg ml^{-1}) and bromocresol green (300 μg ml^{-1}).	This medium was used for enumeration of bifidobacteria from aquatic sources (see Muñoa & Pares, 1988).	Resnick & Levin (1981*b*)
MRS agar with added dicloxacillin and cystein chloridate.	This is a selective medium for enumeration of bifidobacteria in fermented milk products.	Zacconi *et al.* (1990)

required for the selective enumeration of *Bifidobacterium* spp. from dairy products (see Pacher & Kneifel, 1993), and that such development should take into account easy and inexpensive preparation and the ability to achieve > 95% recovery of the organisms.

Direct microscopic counting has been used to enumerate bifidobacteria in fermented milk products (Murti *et al.* 1993). This technique might provide a 'quick' test, for example, to check the ratio of different organisms in yogurt-type products, but does not indicate the viable cell count of bifidobacteria. However, by using the direct epifluorescent filter technique (DEFT), such microscopic tests can distinguish between live and dead cells.

STARTER CULTURE TECHNOLOGY

The traditional scale-up system (stock culture → mother → feeder or intermediate → bulk) of propagating starter cultures for the manufacture of fermented milk products is currently being replaced by DVI of bulk starter or the process milk. Bulk starter milk is prepared by reconstituting antibiotic-free, skimmed milk powder (total solids 100–120 g l^{-1}) and heating to 90–95 °C for 30 or 15 min. This ensures (i) destruction of microorganisms present in milk, (ii) inactivation of natural inhibitors, (iii) partial modification of the proteins and (iv) a reduction in the content of dissolved oxygen. All these factors help to provide the right growth conditions for *Bifidobacterium* spp. However, as shown elsewhere, a growth stimulatory factor(s) is required by the bifidobacteria, and suitable materials can be added to the bulk

starter milk or incorporated with the dry growth medium obtained from the culture suppliers.

No results are available on bacteriophage attack of *Bifidobacterium* spp., or starter failure during the production of the bulk starter or the product on-site. Nevertheless, the use of mechanically protected tanks is highly recommended for the protection of the culture from bacteriophage attack. Different mechanically protected systems have been developed in the dairy industry for the production of starter cultures, and these systems have been described in detail by Heap & Lawrence (1988), Tamime (1990) and Cogan & Hill (1993). Commercially produced bifidobacteria starters give more consistent activity than the in-house production of liquid cultures which are subject to daily transfers; they also maintain the proper balance of mixed cultures when used in combination with *Lb. acidophilus*, *Str. thermophilus* and/or *Ped. acidilactici*. The trend is to use DVI for the bulk starter or process milk.

DVI commercial cultures can be obtained in the concentrated freeze-dried form, or as concentrated cultures deep frozen at −196 °C; the latter can be stored between −40 °C and −80 °C. The former type of culture can be stored at < 5 °C or at −20 °C, and the latter temperature extends the shelf life of the culture. The activity of these cultures is dependent on the survival rate, and the average count is $\geqslant 10^{11}$ cfu g^{-1}. To achieve such high counts, the bacterial cells are concentrated before preservation and storage. These highly concentrated bifidobacteria cultures can also be used as dietary adjuncts for direct consumption, e.g. as pharmaceutical tablets.

There are many different methods that can be used for the concentration of cell biomass of lactic acid bacteria (Tamime, 1990), and the techniques used for concentrating *Bifidobacterium* spp. are mechanical separators (e.g. Sharples), neutralization of growth media at ~ pH 6·0 using 10 M-NH_4OH or 2 M-NaOH and UF or microfiltration (Damjanović & Radulović, 1967; Collins & Hall, 1984; Ishibashi *et al.* 1985; Staab & Ely, 1987; Taniguchi *et al.* 1987*a*, *b*; Misra & Kuila, 1991*b*; Boyaval *et al.* 1992; Corre *et al.* 1992; Gagné *et al.* 1993). The concentrated cultures can be preserved successfully by freeze drying or freezing at low temperatures ranging from −20 °C to −196 °C. Freezing and/or drying can damage the bacterial cells, but the use of cryogenic compounds (e.g. sterile skimmed milk, sucrose, lactose, gelatin, pectin, sodium caseinate, sodium alginate, glycerol and sorbitol) have retained the activity of bifidobacteria. Table 10 illustrates some compounds used for freeze drying different *Bifidobacterium* spp. Factors such as the initial microbial count before preservation, moisture content in the freeze-dried culture, the use of protective compounds, packaging under a controlled atmosphere such as N_2 and using oxygen-impermeable packaging material and the storage temperature can also influence the survival rate of bifidobacteria. A detailed study on the survival rate of *Bifid. infantis* ATCC 27920 after freeze drying using different methods of concentration of the microbial cells and cryogenic compounds has been recently reported by Gagné *et al.* (1993). The same authors concluded that best survival rate was achieved by membrane concentration of the culture, and the addition of skim milk (100 g l^{-1}) and sucrose (120 g l^{-1}) as cryogenic materials.

A wide range of growth media was studied by Corre *et al.* (1992) for the production of concentrated *Bifid. bifidum* TLR 100. A whey-based and low-cost medium was found to be effective for the growth of the organism in a continuous process reactor coupled to a UF unit. Good viability was reported for starters stored at 4, −20 or −80 °C and lyophilized. Bacterial biomass productivity (2×10^3 cfu ml^{-1} h^{-1}) in a continuous process was 15-fold greater than with a batch process.

Table 10. *Selection of different cryogenic compounds employed during the production of freeze-dried* Bifidobacterium *spp.*

Microorganisms	Processing conditions	Survival rate	References
Bifid. longum M-101, ML-3 *Bifid. breve* M-161 *Lactobacillus acidophilus* BR	Grow organism in nutrient culture broth: centrifuge and resuspend in cryoprotecting medium containing sucrose, sodium glutamate and gelatin; freeze dry at −40 °C and 30 °C.	10^{10}–10^{12} cfu g^{-1}	Ishibashi *et al.* (1985, 1990)
Bifid. bifidum ATCC 11146 15696 *Bifid. longum* *Bifid. infantis*	Skimmed milk (SNF, 100–120 g l^{-1}) is fortified with: (a) modified MRS (2 g l^{-1}) or (b) cysteine (5 g l^{-1}) plus pyruvic acid (0·5 g l^{-1}) or ascorbic acid (2 g l^{-1}); grow culture, centrifuge, resuspend in cryoprotecting medium (g l^{-1}): SNF 50. sucrose 80. gelatin 15) and freeze dry (−30 °C and 20 °C).	$6{\cdot}0 \times 10^{8}$ cfu g^{-1}	Collins & Hall (1984)
Bifid. bifidum (three strains) *Bifid. bifidum* NCFB† 1452 1453 *Bifid. bifidum* NDRI‡	Several growth media were studied; centrifuge cultured milk, wash cells with 0·1 M-phosphate buffer and resuspend in sterilized skim milk (SNF, 100 g l^{-1}) plus dimethyl sulphoxide (10 g l^{-1}); freeze dry at −30 °C and 20 °C.	10^{10}–10^{11} cfu g^{-1}	Misra & Kuila (1991*b*)
Bifid. infantis ATCC 27920	The culture was grown in commercial MRS medium containing lactose (25 g l^{-1}) at constant pH 6·0 using 10 M-NH_4OH and temperature maintained at 37 °C: after ~ 12 h fermentation. the cells were harvested by centrifugation, microfiltration or ultrafiltration and suspended in three cryoprotective media containing skim milk. sucrose and gelatin or combination of these ingredients plus ascorbic acid and freeze dried at −40 °C and 30 °C for 12 h followed by 4 h at 40 °C.	See text	Gagné *et al.* (1993)

† National Collection of Food Bacteria. UK (formerly National Collection of Dairy Organisms. NCDO).
‡ National Dairy Research Institute. India.

The same group of researchers (Boyaval *et al.* 1992) used a two stage cell-recycle bioreactor plant for the continuous production of concentrated *Lb. acidophilus* TR1 101 and *Bifid. bifidum* TRL 100 without mixing the cultures. The growth medium used was sweet cheese whey fortified with autolysed yeast extract and ascorbic acid. The cell productivities of *Lb. acidophilus* and *Bifid. bifidum* were $7{\cdot}6 \times 10^{7}$ and $3{\cdot}2 \times 10^{7}$ cfu ml^{-1} h^{-1} respectively.

FUTURE DEVELOPMENTS

Current scientific research and the manufacture of dairy products as dietary adjuncts is mainly centred in Japan and a few countries in Europe. Their future status in the USA is dependent on some barriers being overcome, such as poor consumer awareness of the possible health benefits of using bifidobacteria in dairy products,

and the need for the addition of *Bifidobacterium* spp. to face US regulatory scrutiny by the Food and Drug Administration (Hoover & Hughes, 1991). Nevertheless, the review by Renner (1991) points out that cultured dairy products containing *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Pediococcus* spp. and, in some instances, *Enterococcus faecium* are beneficial for human health. In general, these organisms should

originate from a human source and be non-pathogenic,
be capable of surviving and growing in the intestinal tract,
have the ability to attach to gut epithelial cells or mucus,
be able to produce beneficial effects in the gut and
be viable, active and abundant in the product before consumption.

Taking these aspects into account, possible future developments in this field may include:

selection of suitable strains capable of fermenting milk within an acceptable period of time,
wider application of bifidobacteria in the dairy industry,
genetic manipulation, if possible, to enhance their growth in milk, and to reduce the high amounts of acetic acid produced,
since *in vitro* studies cannot be always related to *in vivo* trials, or the results of animal experiments be applicable to human beings, improvements in the design of experiments together with trials involving human volunteers,
greater involvement of the medical profession in studying the beneficial effects of therapeutic microorganisms,
improved funding of bifidobacteria research and greater collaboration between scientists, nutritionists and technologists in many countries, and
starter cultures that depend on each other and inhibit the growth of undesirable microorganisms.

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D130

Morphological Variability in the Bifidobacteria

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With the increasing employment of bifidobacteria in dairy products, the ability to monitor viable cell counts has become of major importance. However, checking isolated colonies to confirm identity has proved difficult, because the morphology of the bifidobacteria can be extremely variable. Thus, an isolate of Bifidobacterium bifidum from a commercial source was, on Rogosa's Modified Medum, morphologically indistinquishable from Streptococcus sp. but, on plating dilutions of these colonies onto a Minimal Medium, many of the resultant colonies contained bacteria with the typical branched structure. In other words, the same strain of Bif. bifidum appeared either branched or coccoid depending upon the substrate.

SURVIVAL OF *BIFIDOBACTERIUM BIFIDUM* IN 'HEALTH-PROMOTING' YOGHURTS

by

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ABSTRACT

If the 'health-promoting' reputaton of bifidus yoghurts/desserts is to be maintained, then it is essential that the products always contain the 'therapeutic minimum' number of viable cells of *Bifidobacterium* spp. at the time of consumption. In particular, the potentially adverse effect of certain types of popular flavouring ingredient should be monitored, as should the possible influence of other starter bacteria that may be present.

Introduction

Over the last few years, the medical profession has become increasingly sympathetic to the view that the composition of the intestinal microflora can have a profound influence on the health of an individual. In particular, the important role of bifidobacteria has become widely accepted, as has the function of diet in both the destruction and replenishment of this essential component. One conclusion that may be drawn from the literature is that fermented dairy products could play a crucial role in the maintenance of the natural microflora of the gut (Klupsch, 1985; Robinson, 1989). This beneficial activity may involve either stimulation of the growth/metabolism of the existing microflora, or the establishment of the ingested bacteria, notably bifidobacteria, if the indigenous population has been reduced by antibiotic therapy or other inhibitory factor (Hotta *et al*, 1987). Whatever the ultimate interaction between the indigenous and ingested bacteria, the belief is growing that the regular consumption of a fermented milk, e.g. one carton (150 g) of product including *Bifidobacterium bifidum* or *Bif. longum* with a minimum viable count of 10 x 10^5/mℓ, will be a beneficial impact on the physiology of the consumer.

One effect of this widespread acceptance is that sales of yoghurts (including Mild Yoghurts — so designated to indicate the absence of *lactobacillus bulgaricus)* containing a 'health-promoting' culture now account for 20 % of the total market in some European countries. Maintenance of this position does depend, of course, on continuing consumer belief in the product, a belief that will be severely strained if ineffective products arrive on the market, i.e. yoghurts with few viable bacteria of starter origin. Nevertheless, retail outlets will continue to demand a greater range of products, and most likely, in the area of health-promoting yoghurts with added fruit/flavours — most existing brands are natural. Some Mild Yoghurts with added fruit/flavour are available, but the advantages of producing yoghurt *per se* are:

(i) Consumers are familiar with characteristics of the standard, stirred fruit yoghurt, and will readily accept new varieties that are similar:
(ii) The manufacturing procedure need be little different from that for normal yoghurt; and
(iii) The final acidity of the yoghurt will be around pH 4,0 — 4,1, and hence there should be no risk to the public from *listeria* or other pathogens.

However, low pH does affect adversely the survival of many strains of bifidobacteria, and it is essential that manufacturers check that products do contain the desired minimum number of viable bifidobacteria throughout their predicted shelf-life.

The aim of the present work was to confirm this point, and in particular, to study:

(i) the effect of a range of commercial fruit purées and flavouring materials on the survival of *Bif. bifidum;* and
(ii) whether the presence of a yoghurt culture consisting of *Streptococcus thermophilus* and *Lac. bulgaricus* would influence the survival of the bifidobacteria.

Materials and Methods

Freeze-dried cultures of *Bif. bifidum* and a yoghurt culture (Type B3) were obtained from Chr. Hansen's Laboratorium A/S, Denmark, and after resuscitation, the working cultures were held in skim-milk (12 % total solids (TS) with weekly transfers.

The base for the bifidus dessert, i.e. no yoghurt culture, was reconstituted skim-milk powder (14 % TS), which after recombination with a high-shear mixer, was heat treated at 85 °C for 30 minutes. The milk was then cooled to 37 °C and inoculated with a fresh culture of *Bif. bifidum* at the rate of 2 % v/v. After thorough mixing, the milk was incubated at 37 °C for 19 hours to

achieve a pH 4,0. These two figures were selected as representing a Mild Yogurt (pH 4,5) and a normal yoghurt (pH 4,0-4,1), for although the optimum pH for the survival of this strain in milk was found to be 5,0, the product was too mild to merit serious consideration. At the selected acidities, the two batches of product were cooled overnight at 4 °C.

Each batch was then divided into seven lots of 1,5 litres, and while one was held as a control, three received approximately 150 g of either Banana, Black Cherry or Strawberry purée, and three were blended with 150 g of an equivalent sugar/starch base flavoured with Chocolate, Mocha or Toffee. These fruits/flavouring materials were standard commercial products obtained from G.R. Spinks & Co., Devon (UK), and hence no attempt was made to modify the composition. The final pHs of the products were checked, however, and if necessary adjusted with lactic acid or calcium hydroxide to the desired figure.

The procedure employed to prepare the bifidus yoghurts was identical to that described above, except that the inoculum consisted of 1,5 % *Bif bifidum* and 0,5 % of yoghurt culture. The incubation times required to obtain products with final pH values of 4,5 and 4,0 were three and five hours respectively, due in the main to the activity of the yoghurt culture. After cooling, the bifidus yoghurts were blended with the same range of fruits/flavours. At this point, the various lots of bifidus dessert and yoghurt were dispensed into polypropylene cartons (175 g) with push-on lids. Duplicate cartons of each lot were sampled immediately in order to record the total number of viable bifidobacteria, and the procedure was repeated at seven, 14 and 21 days, along with measurements of pH. Enumeration involved making dilution series in quarter-strength Ringer's solution, followed by duplicate plating (pour plate method) onto Tomato Juice Agar (*Bif. bifidum* alone) or Rogosa's Selective Agar where suppression of the yoghurt bacteria was desired (Rasic & Kurmann, 1983). In both cases, the plates were incubated anaerobically in an atmosphere of hydrogen and carbon dioxide (95:5) at 37 °C. After four days, the colonies were sufficiently developed for counting, and typical examples were checked microscopically to ensure the absence of yoghurt organisms. The results were recorded as mean counts for each pair of cartons.

Results and Discussion

The pHs of the bifidus desserts were, as would be expected from previous work (Rasic & Kurmann, 1983), comparatively stable throughout the period of storage, and the final values for batches never fell below 3,9 (from 4,0) or 4,4 (from 4,5). Nevertheless, even these values are low in relation to the optimum preferred by *Bif. bifidum*, and yet the figures for survival shown in Table 1 show remarkable stability. The impermeability of the cartons to oxygen may have encouraged the observed viability, but even so the strain does appear extremely tolerant of acid conditions. It is of note also that none of the fruit/flavours adversely affected the situtation, and hence there would seem to be no reason why bifidus desserts cannot be tailored to meet consumer demand. Obviously, the actual figures at $> 1 \times 10^9$ of *Bif. bididum* would be too high for a commercial product — at these levels, the presence of acetic acid gives the product an unacceptable flavour — but the results do suggest that desserts with 10^8 — 10^7 cell/g of product (at the time of consumption) could be manufactured without too many problems over residual counts.

TABLE 1. Survival of *Bif. bifidum* in bifidus desserts with the initial pHs indicated; all figures as cfu/g of product

Variety	Days			
	0	21	0	21
	(pH 4,0)		(pH 4,5)	
Natural	$4,5 \times 10^9$	$3,5 \times 10^9$	$4,8 \times 10^9$	$3,5 \times 10^9$
Banana	$3,9 \times 10^9$	$3,2 \times 10^9$	$4,2 \times 10^9$	$3,4 \times 10^9$
Black Cherry	$4,3 \times 10^9$	$3,7 \times 10^9$	$4,1 \times 10^9$	$4,2 \times 10^9$
Strawberry	$3,3 \times 10^9$	$3,5 \times 10^9$	$2,8 \times 10^9$	$3,8 \times 10^9$
Chocolate	$3,2 \times 10^9$	$3,2 \times 10^9$	$4,3 \times 10^9$	$3,9 \times 10^9$
Mocha	$3,7 \times 10^9$	$3,2 \times 10^9$	$4,1 \times 10^9$	$3,2 \times 10^9$
Toffee	$3,2 \times 10^9$	$2,9 \times 10^9$	$4,9 \times 10^9$	$3,8 \times 10^9$

However, as mentioned earlier, a more attractive route would be to produce a bifidus yoghurt, but as that data in Table II indicate, the process conditions need to be selected with care. The inhibitory effect of the yoghurt culture is evident, especially when the bifidobacteria are stressed by low pHs. Thus, with an initial pH of 4,0, a value of 3,8 was reached after only seven days — as against 14 days for the batch at pH 4,5 — and these contrasted degrees of exposure appear to be reflected in the final counts for the natural product.

TABLE 2. Survival of *Bif. bifidum* in bifidus yoghurts with the initial pHs indicated; all figures as cfu/g of product

Variety	Days			
	0	21	0	21
	(pH 4,0)		(pH 4,5)	
Natural	$2,0 \times 10^7$	$1,8 \times 10^4$	$1,9 \times 10^7$	$9,7 \times 10^6$
Banana	$2,0 \times 10^7$	$1,7 \times 10^4$	$1,6 \times 10^7$	$6,1 \times 10^5$
Black Cherry	$1,8 \times 10^7$	$6,5 \times 10^5$	$5,2 \times 10^7$	$1,0 \times 10^7$
Strawberry	$1,7 \times 10^7$	$4,3 \times 10^5$	$3,1 \times 10^7$	$4,2 \times 10^6$
Chocolate	$2,3 \times 10^7$	$< 10^2$	$2,4 \times 10^7$	$9,0 \times 10^3$
Mocha	$1,7 \times 10^7$	$2,3 \times 10^2$	$1,1 \times 10^7$	$2,0 \times 10^3$
Toffee	$3,4 \times 10^7$	$1,8 \times 10^3$	$1,9 \times 10^7$	$9,7 \times 10^5$

Whether this adverse influence of the yoghurt culture is due to the generation of hydrogen peroxide (Hamman & Marth, 1984) is not clear, but certainly *Bif. bifidum* is catalase negative. One solution to this problem might be to lower the inoculation rate of the yoghurt culture to < 0,1 %, for at this reduced level it should be possible to gain the advantages of rapid acidification during production without the deleterious effect on the bifidobacteria (Anon, 1985). The combined stresses of low pH and competition from the yoghurt culture also revealed the need to monitor the potential influence of flavouring compounds. Thus, in both batches, chocolate and mocha depressed the levels of bifidobacteria well below the therapeutic minimum, and with the low pH batch, the figures had fallen to 1×10^4 within seven days. The impact of the fruits was of less concern, and certainly with yoghurt produced to a pH of 4,5, any of the three fruits could be employed. Other fruits might, of course, give rise to a different reaction, but so long as a manufacturer is prepared to standardise the production procedure in terms of inoculum size and final acidity, and monitor the situation with regard to the survival of bifidobacteria, then stirred, fruit, bifidus yoghurts could become a common sight in the supermarket.

The potential advantages of incorporating bifidobacteria into already fermented yoghurt also deserves appraisal, for although this route offers, in theory, a most attractive option, i.e. high counts, low cost and little risk of off-flavours, it will still be necessary for the viability of the selected strain to be proven under normal conditions of storage/distribution.

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ORIGINAL ARTICLE

Acid production by bifidobacteria and yoghurt bacteria during fermentation and storage of milk

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Three species of bifidobacteria, namely Bifidobacterium bifidum, Bifidobacterium longum *and* Bifidobacterium adolescentis *were used in pure culture and in combination with yoghurt bacteria (B3 and SBI cultures) for the production of fermented milks. The number of bacteria during fermentation and the level of acid produced during fermentation and storage were assessed using Rogosa's modified selective agar and high performance liquid chromatography (HPLC). It was found that during fermentation all bifidobacteria exhibited growth uncoupled from acid production. Two of the species examined produced only low levels of acids when grown individually and only* B. adolescentis *produced appreciable amounts. In mixed cultures, the level of acid was a reflection of the combination of yoghurt culture and species of* Bifidobacterium, *and this, observation suggests that there is a degree of influence between the cultures. During storage, the acid concentration remained quite stable in most samples. The prevention of post-production acidification that normally occurs during storage of yoghurt can be attributed to the presence of bifidobacteria, and it could be that acetic acid has a marginally inhibitory effect on the* Lactobacillus *and* Streptococcus *spp.*

.ntroduction

Bifidobacterium species are one of the major components of the intestinal flora of healthy humans, and Tissier (1906) was the first to promote the therapeutic use of the bifidobacteria for the relief of digestive disorders. He believed that the bifidobacteria displaced the putrefractive bacteria, which are responsible for gastric upsets while, at the same time, re-establishing themselves as the dominant intestinal organisms. This rationale is very similar to the better known arguments of Metchnikoff in his 'longevity' theory (Metchnikoff 1908). In order for bifidobacteria to exert their beneficial effect in the host, they have to be alive in the product at the time of consumption, and be capable of reaching the intestine. When they have established themselves, they can then multiply and produce acids which: (1) lower the pH of the colon; (2) inhibit the growth of the undesirable organisms; and (3) increase intestinal peristalsis, which helps to remove any potential pathogens.

Although cultured milks containing bifidobacteria possess many nutritional and technological advantages, such as limited post-production acidification, a mild taste, the formation of the physiologically desirable L(+)lactic acid, and the therapeutic benefits

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that follow the consumption of 'live' bifidobacteria, there are still many problems associated with the manufacture of such products.

One of the most important is slow acid production, which results in a prolonged fermentation time. The dairy industry faces this problem by using combined cultures of bifidobacteria and other lactic acid bacteria. Some manufacturers employ *Streptococcus thermophilus* alongside the bifidobacteria, but a number of 'bifidus' products are fermented using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.

The advantage of using a mixed culture containing bifidobacteria and yoghurt bacteria is not only the reduction of the fermentation time, but also the avoidance of other defects that fermented products containing only bifidobacteria may have, such as whey separation, sandy or slimy texture, too mild taste, yeasty or vinegary taste or too little aroma (Rasic and Kurmann 1983). In addition, the addition of yoghurt cultures to 'bifidus' products may improve their dietetic value (Gilliland 1991).

However, Samona and Robinson (1994) found that some strains of yoghurt bacteria could have an adverse effect (reduction of growth) on some species of *Bifidobacterium*, and Robinson (1988) found a similar interaction in flavoured yoghurt-like products. The cause(s) and effect(s) of this interference were not established, and hence the purpose of this present work was to examine the influence of certain yoghurt bacteria on the metabolism of lactose by bifidobacteria during fermentation and storage.

Materials and Methods

Organisms

The bifidobacteria were *Bifidobacterium bifidum* and *Bifidobacterium longum* supplied in freeze-dried form by Chr. Hansen's Laboratories Ltd., Reading, UK and *Bifidobacterium adolescentis* isolated from commercial products. Identification to generic level was confirmed by morphology and enzymic tests, while the pattern of sugar fermentations was employed to identify the species (Samona and Robinson 1991).

The yoghurt culture designated B3, was obtained from Chr. Hansen's Laboratories Ltd., and culture SBI was supplied by Sanofi Bio-Industries, Newbury, UK. The yoghurt cultures were separated in the laboratory into single species, namely *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, and because of their origin, no further identification steps were considered necessary. All the yoghurt cultures were obtained in a freeze-dried form.

Culture propagation and maintenance

The freeze-dried cultures were activated in reconstituted skim-milk (12% total solids) heat treated at 115°C for 1 min, and the yoghurt cultures were then plated onto Lee's Medium (Lee et al. 1974) to separate the individual species. After this initial resuscitation and separation, the cultures were maintained in skim-milk at 4°C, and each week, an aliquot from each culture (*c.* 5% v/v) was transferred to freshly prepared milk. The inoculated milk was then incubated at 37°C until the pH had dropped to about 4·9–5·0, and the fresh cultures were stored at 4°C until required.

Inoculation of the test milks

The milk used in the experiments was reconstituted skim-milk (12% total solids) heat treated at 85°C for 30 min to simulate commercial operation. The control milks were inoculated with pure cultures of *Bifidobacterium*, and an inoculum of 4% (v/v) was used. However, for the experiments with the mixed cultures, an inoculum of 3% (1% of each of the three species) was used, and Table 1 shows the combinations of bacteria that were examined.

Design of the experiment

Batches of milk (500 ml) were inoculated with the combinations of cultures shown in Table 1, or the single cultures of bifidobacteria, and sub-samples (10 ml) were taken at 0, 6, 12, 24 and 48 h; with the mixed cultures,

Table 1. Various combinations of starter bacteria that were grown together during the course of this work, alongside pure cultures of the *Bifidobacterium* spp.

Live culture of *Bifidobacterium*	Starter bacteria	
Bifidobacterium adolescentis	+ B3 Str	+ B3 Lb
Bifidobacterium adolescentis	+ SBI Str	+ SBI Lb
Bifidobacterium bifidum	+ B3 Str	+ B3 Lb
Bifidobacterium bifidum	+ SBI Str	+ SBI Lb
Bifidobacterium longum	+ B3 Str	+ B3 Lb
Bifidobacterium longum	+ SBI Str	+ SBI Lb

Str, *S. thermophilus*; Lb, *L. delbruekii* subsp. *bulgaricus*.

testing was halted at 24 h, because it was anticipated that the continued development of acidity by the yoghurt cultures would inhibit any activity by the bifidobacteria. The samples were used to determine the total colony counts of bifidobacteria (cfu ml^{-1} milk), and for determination of the levels of lactic acid, acetic acid and galactose present in the fermented products. For the evaluation of the organic acids during the storage trials, the fermentation was stopped when the pH of the product was 4·6 and samples were analysed after 0, 7, 14 and 21 days under refrigeration.

Experimental procedures

Microbiological. For the enumeration of bifidobacteria, samples (1 ml) were withdrawn from the relevant sub-samples (10 ml), and serial dilutions to 10^{-8} were made in 0·1% peptone diluent. Duplicate pour plates were then set-up from the three highest dilutions with Rogosa's modified selective agar (RMSA) as the medium (Samona and Robinson 1991). The plates were incubated anaerobically at 37°C for 72 h, and the mean total colony counts recorded as cfu ml^{-1} of the original milk; the dilution giving rise to counts between 25–250 was selected for counting.

Analysis by high pressure liquid chromatography HPLC. The liquid chromatography system consisted of a Spectra-Physics HPLC apparatus, an Isochrom isocratic pump, an Altex model 210 injector with a 50 µl loop, a model 100 ultra violet (UV) absorbance detector, an 8430 refractive index (RI) detector and an SP4400 dual-channel intergrator. The RI detector was operated at a sensitivity of $0{\cdot}020\times10^{-3}$ units and a response time of 2·0 units. The UV detector was operating at the 210 nm wavelength, with a rise time of 1 s and a sensitivity of 0·01 absorbance units (full scale). The peak threshold value of the intergrator was set at 50.

The UV detector was used for the determination of the organic acids, and the RI detector for the determination of galactose. The separation of the compounds was carried out on a Bio-Rad Aminex HPX 87H cation exchange column, with a cation H^+ guard column. The mobile phase was 0·01 N H_2SO_4, and the analyses were performed at a flow rate 0·6 ml min^{-1} and at a temperature of 45°C.

Samples (1 ml) were withdrawn from the relevant sub-samples (10 ml) of the fermented milks, and were pipetted into 100 ml volumetric flasks. After dilution with distilled water to 100 ml, each sample was ultra-filtered, at refrigeration temperature, using a membrane with a molecular weight cut-off of 1000. The ultra-filtered samples were collected into 5 ml sterile containers and stored in a freezer (–30°C). Duplicate aliquots of each sample were injected into the HPLC system, so that the results are means of two runs.

Results and Discussion

The rates of growth and acid production by the pure cultures of *Bifidobacterium* sp. are shown in Tables 2, 3 and 4. The accumulation of galactose in the medium suggests that, although the species are galactose-positive, the glucose portion of lactose was being metabolised preferentially. *B. adolescentis* produced 10 times more acid than the other two species, and it was of note that lactic acid

Table 2. Total colony counts (cfu×10^6 ml^{-1} of milk) of *Bifidobacterium adolescentis* when grown alone or in conjunction with the yoghurt cultures indicated.

Time (h)	*Bifidobacterium adolescentis*				*Bifidobacterium adolescentis*+B3				*Bifidobacterium adolescentis*+SBI			
	Count	Acetic acid	Lactic acid	Galactose	Count	Acetic acid	Lactic acid	Galactose	Count	Acetic acid	Lactic acid	Galactose
0	140	0·0	0·0	0·0	50	0·0	0·0	0·0	50	0·0	0·0	0·0
6	130	1·0	45·0	25·0	77	2·0	40·0	15·0	61	9·0	40·0	15·0
12	670	1·4	135·0	65·0	37	1·4	80·0	40·0	75	5·0	85·0	25·0
24	370	1·4	180·0	85·0	43	1·4	160·0	60·0	71	4·0	95·0	25·0
48	410	1·4	290·0	140·0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

The levels of acetic acid, lactic acid and galactose (mmol l^{-1}) detected in the same milks after incubation at 37°C for the times specified are shown as well.
n.d. not determined.

Table 3. Total colony counts (cfu×10^6 ml^{-1} of milk) of *Bifidobacterium bifidum* when grown alone or in conjunction with the yoghurt cultures indicated

Time (h)	*Bifidobacterium bifidum*				*Bifidobacterium bifidum*+B3				Bifidobacterium bifidum+SBI			
	Count	Acetic acid	Lactic acid	Galactose	Count	Acetic acid	Lactic acid	Galactose	Count	Acetic acid	Lactic acid	Galactose
0	270	0·0	0·0	0·0	50	0·0	0·0	0·0	50	0·0	0·0	0·0
6	240	1·0	1·0	0·2	60	1·0	50·0	25·0	40	8·0	5·0	5·0
12	280	12·0	9·0	0·3	67	1·0	90·0	45·0	50	0·2	30·0	15·0
24	270	15·0	14·0	0·3	40	16·0	225·0	80·0	50	0·0	50·0	25·0
48	200	35·0	35·0	0·4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

The levels of acetic acid, lactic acid and galactose (mmol l^{-1}) detected in the same milks after incubation at 37°C for the times specified are shown as well.
n.d. not determined.

Table 4. Total colony counts (cfu×10^6 ml^{-1} of milk) of *Bifidobacterium longum* when grown alone or in conjunction with the yoghurt cultures indicated

Time (h)	*Bifidobacterium longum*				*Bifidobacterium longum*+B3				Bifidobacterium longum+SBI			
	Count	Acetic acid	Lactic acid	Galactose	Count	Acetic acid	Lactic acid	Galactose	Count	Acetic acid	Lactic acid	Galactose
0	600	0·0	0·0	0·0	50	0·0	0·0	0·0	50	0·0	0·0	0·0
6	430	1·0	1·2	0·4	48	5·0	65·0	40·0	20	5·0	20·0	9·0
12	330	9·0	9·0	0·4	39	9·0	160·0	85·0	30	8·0	20·0	8·0
24	430	12·0	12·0	0·4	20	9·0	210·0	98·0	40	8·0	65·0	20·0
48	450	25·0	25·0	0·4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

The levels of acetic acid, lactic acid and galactose (mmol l^{-1}) detected in the same milks after incubation at 37°C for the times specified are shown as well.
n.d. not determined.

was the major metabolic product. The attraction of this extreme imbalance is that the risk of a 'vinegar' taint in a product as a result of the acid produced is unlikely to arise, and the acetic acid may still be secreted in sufficient quantity *in vivo* to have a beneficial effect (antimicrobial effect, inhibition of the growth of undesirable bacteria). Although an appreciable quantity of lactic acid was produced by *B. adolescentis* during the maximum phase of growth (0–12 h), a high level of acid was produced after the increase in cell count had ceased (24–48 h); so enhancing the idea that bifidobacteria can exhibit uncoupled growth and acid production. *B. bifidum* and *B. longum* produced very low levels of the acid under the conditions employed, and hence it is not surprising that these cul-

Table 5. Levels of acetic and lactic acids and galactose (mmol l^{-1}) detected during storage of milks at 4°C

Time (days)	*Bifidobacterium adolescentis*+B3			*Bifidobacterium adolescentis*+SBI			*Bifidobacterium bifidum*+B3			*Bifidobacterium bifidum*+SBI			*Bifidobacterium longum*+B3			*Bifidobacterium longum*+SBI		
	Galactose	Acetic acid	Lactic acid	Galactose	Acetic acid	Lactic acid	Galactose	Acetic acid	Lactic acid	Galactose	Acetic acid	Lactic acid	Galactose	Acetic acid	Lactic acid	Galactose	Acetic acid	Lactic acid
0	39·7	5·9	105·9	35·3	5·9	98·5	35·9	7·4	102·9	36·8	2·9	88·2	36·9	7·4	73·5	25·0	5·6	60·3
7	42·6	8·8	105·9	32·4	7·4	89·7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	33·8	8·8	100·0	n.d.	n.d.	n.d.
14	45·6	8·8	102·9	38·2	5·9	94·1	36·8	7·4	85·3	50·0	8·8	119·1	45·6	7·4	105·9	26·5	5·6	54·4
21	44·1	10·3	107·4	39·7	4·4	95·6	38·2	8·9	105·9	52·9	8·8	122·1	48·5	8·8	105·9	27·9	8·8	61·8

n.d. not determined.

tures are rarely employed alone in commercial practice.

During the incubation of the mixed *Bifidobacterium* and yoghurt cultures, the total acidity produced by the mixed cultures was, with the exception of *B. adolescentis*, higher than that released by the pure cultures of bifidobacteria.

All the mixed cultures produced, as expected, more lactic than acetic acid, but culture (SBI) did appear to stimulate the release of acetic acid by *B. adolescentis* and *B. bifidum* during the first 6 h. In all cases, galactose tended to accumulate as the level of lactic acid increased, so indicating that the bacteria were using glucose for their metabolism.

In the experiments with *B. adolescentis* (Table 2), the rate of lactic acid production slowed after 12 h of incubation in the samples containing the SBI culture, whereas it continued to increase in the samples with the B3 culture; the major increase in the latter sample occurred between 12 and 24 h of incubation.

With *B. bifidum* and *B. longum*, high levels of acid were produced when the bacterium was combined with the B3 yoghurt culture, whereas lower levels were recorded with the SBI culture (Tables 3 and 4).

Comparing the counts of *B. bifidum* and *B. longum*, it is noticeable that, whereas *B. bifidum* was unaffected by the presence of the yoghurt cultures, the numbers of *B. longum* showed a tendency to decline. *B. adolescentis* was similarly resistant to any influence from the yoghurt cultures, but unlike the situation with the pure culture (Table 2), there was no detectable increase in cell count. This pattern is broadly in line with that reported elsewhere (Samona and Robinson 1994), in that whilst bifidobacteria may not grow in the presence of yoghurt cultures, only certain combinations of strains lead to an actual decline in cell counts.

This post-production stability was revealed also by the levels of galactose, lactic acid and acetic acid detected during the storage of milks fermented with bifidobacteria and yoghurt cultures (Table 5). Overall, the storage of the fermented milks caused little change in the acid levels. When the B3 yoghurt culture was used, there was some increase in the lactic acid content only in the sample that contained *B. longum*, whereas with the SBI yoghurt culture, this increase occurred when the *B. bifidum* was used. *B. adolescentis* kept the amounts of acids produced quite stable with both yoghurt cultures.

Conclusions

Two of the *Bifidobacterium* species examined produced only low levels of lactic acid and acetic acids during incubation alone at 37°C, but *B. adolescentis* produced lactic acid in appreciable amounts; this species resembled the Biogarde species described by Chevalier et al. (1990). All three species appeared to have uncoupled growth and acid production.

In mixed yoghurt and *Bifidobacterium* cultures, the levels of acid were a reflection of the combination of yoghurt culture and species of *Bifidobacterium*, and this observation suggests that there is a degree of interference between the cultures.

The nature of the interaction was not established but, because the higher levels of acid were obtained with yoghurt culture (B3) and the lower with the SBI, it appears that the main pattern of acid production is controlled by the yoghurt bacteria. The low level

of acid present in the samples that contained the SBI culture could be attributed, perhaps, to a selected incompatibility between the constituent species of yoghurt bacteria, i.e. chosen to give a mild yoghurt and little post-production acidification.

During storage, the acid concentration remained quite stable in most samples (Table 5). This prevention of the late-acidification that usually occurs during the storage of yoghurt could be attributed to the presence of bifidobacteria, and it could be that the acetic acid has a marginally inhibitory effect on the lactobacilli and/or streptococci. If this effect is widespread, then it could help to explain the fact that a combination of bifidobacteria with other lactic acid bacteria can give a mild product with an extended shelf life.

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ORIGINAL CONTRIBUTION

Enumeration of bifidobacteria in dairy products

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There is much interest in the potential role of bifidobacteria as dietary inclusions, but their isolation from dairy products, and subsequent identification, can cause problems. A range of selective and non-selective media were examined, and modified Rogosa agar was found to give the best recovery from yogurt-like products. A morphological examination of typical isolates suggested that only bifidobacteria had grown on the medium, but confirmation by means of biochemical tests proved inconclusive, and it seems that the genus/species definition merits further attention.

INTRODUCTION

Bifidobacteria were first observed by Tissier (1899, 1900), who named the organism *Bacillus bifidus*, and they have since been described by Norris *et al* (1950), Schuler *et al* (1969), Sandine *et al* (1972), Poupard *et al* (1973) and Kandler & Lauer (1974). They are Gram-positive, anaerobic, non-spore forming rods of variable appearance (Kurmann and Rasic, 1983) and, recently, the genus has attracted a great deal of attention.

There is a considerable body of evidence to suggest that the regular consumption of milks fermented with bifidobacteria can be beneficial to human health (Robinson, 1989). However, it is equally clear that, in order for these advantages to become manifest, the product has to meet certain clearly defined criteria and, in particular:

(i) The strains of bifidobacteria must be of human origin;

(ii) the strains should possess demonstrable resistance to the conditions encountered within the digestive tract, eg, presence of bile salts; and

(iii) the cells must remain viable throughout the projected shelf-life of the product, so that the item will, at the time of consumption, contain a minimum level of at least 10×10^5 active cells/g.

The former requirements can be met without difficulty by any reputable supplier of starter cultures, but the latter aspect is clearly the responsibility of the manufacturer of the retail product. In the past, this need to monitor the survival of the starter organisms has been often neglected, with the result that a number of product lines have reached the market with few viable bacteria. The inevitable failure of such products to possess any therapeutic properties has made many consumers totally cynical about the entire concept, and only by the studious avoidance of the sale of dubious products can manufacturers hope to regain the confidence of potential purchasers.

If the fermented milk contains bifidobacteria alone, then enumeration onto a selective medium should not pose too many problems (Kurmann & Rasic, 1983) but, today, there is an increasing tendency to introduce other desirable bacteria as well, such as *Lactobacillus acidophilus* and/or a yogurt culture composed of *Lactobacillus delbrueckii* subsp *bulgaricus* (*L bulgaricus*) and *Streptococcus salivarius* subsp *thermophilus* (*S thermophilus*). There are a number of advantages in following this trend, including both health benefits for the consumer (Chandan, 1990), and ease of manufacture (Robinson & Tamime, 1990), but monitoring the end products for the presence of viable bifidobacteria becomes a much more demanding procedure.

The problems arise because few media are truly selective for bifidobacteria alone, and hence the aims of this project were: (i) to examine a range of possible media to determine which could be employed, with or without modification, to selectively enumerate bifidobacteria in the presence of other lactic cultures; and (ii) to ascertain whether the medium selected above would perform equally well for the commercially important species of bifidobacteria.

MATERIALS AND METHODS

Organisms

Species isolated from commercial products, as well as a freeze-dried culture of *Bifidobacterium bifidum* provided by Chr Hansen's Laboratories Ltd, 476 Basingstoke Road, Reading, Berkshire, were used in the experiments.

After the initial isolation, the cultures were maintained in sterile skimmed milk (12% total solids) at 4°C. Each week an aliquot from each culture was transferred to fresh milk (2% v/v) and incubated for 20-24 hours at 37°C. This culture was then stored at 4°C prior to use and/or subculturing.

Media

The non-selective media examined were: (a) Tomato juice agar (TJA), (b) Tomato casein peptone yeast autolysate agar (Orla-Jensen, 1943), (c) BL-agar (glucose-blood-liver agar) (Ochi *et al*, 1964, modified by Teraguchi *et al*, 1978), (d) Rogosa's modified agar (RM agar) (Shimada *et al*, 1977), (e) Tryptone phytone yeast extract agar (TPY agar) (Scardovi, 1986) and (f) MRS (Oxoid).

The selective media examined were: (a) selective modified Rogosa's agar (RMS agar) (Shimada *et al*, 1977), (b) TPY selective agar, (c) MRS selective agar, (d) BL-selective agar (Shimada *et al*, 1977).

Details of the selective solutions added to each of the

selective media are given in Table 1; the concentrations were chosen on the basis of a number of preliminary trials.

TABLE 1
Amount and composition of the selective solutions used for each (selective) medium

Medium	*Selective solution (ss)*	*Amount of ss/100 ml of medium*
Rogosa's agar	PPNL*	4.0 ml
Tryptone phytone yeast extract-S agar	NNL†	5.0 ml

*PPNL: sodium propionate 60% w/v; paromomycin sulphate 0.2% w/v; neomycin sulphate 0.8% w/v; lithium chloride 12% w/v.
†NNL: neomycin sulphate 0.2% w/v; nalidixic acid 0.03% w/v; lithium chloride 6% w/v.

Determination of microbial numbers

The number of viable micro-organisms in each sample was determined by the pour plate method (Harrigan & McCance, 1976). A laser counter (Spiral System Instruments, model 500-A) was used, and random visual counts were made to ensure that there was no interference from the media.

The plates were incubated anaerobically at 37°C for 3 days. Anaerobic conditions were achieved using the Oxoid system, ie, a polycarbonate jar (3 litre) enclosing a gas generating kit capable of generating approximately 1800 ml hydrogen and 350 ml carbon dioxide.

Examples of the characteristic morphology of *Bifidobacterium* spp. *Top*: The typical branched structure. *Bottom*: The rod form, with some rods showing the bone like feature.

Morphological examination

Colonies selected at random for confirmatory purposes were Gram stained and examined under a light microscope. Photographs of some typical bifidobacteria are shown in the Figure, and the characteristic morphology was employed, in part, for purposes of identification. In addition, cells harvested from TPY broth were disrupted using the Hughes press (Hughes, 1951), and the fructose-6-phosphate phosphoketolase test was applied to the cellular extracts, according to the instructions of Scardovi (1986).

Bifidobacterium is alleged to be the only genus of the lactic acid bacteria that gives a positive reaction in this test, since it is the only one that degrades glucose via the fructose-6-phosphate shunt, in which fructose-6-phosphate phosphoketolase cleaves fructose-6-phosphate to give rise to the precursors of lactic and acetic acids. However, there are species of bifidobacteria that do not give a positive reaction in this test (Chevalier *et al*, 1990), and further testing was needed to prove the genus. For this reason the enzymic profiles of three species were determined using the API ZYM test. According to the results obtained by various researchers (Desjardins & Roy, 1990), almost all bifidobacteria possess α- and β-galactosidase and β-glucosidase activities. However, there are some strains of bifidobacteria that give negative results to both fructose-6-phosphate phosphoketolase and API ZYM tests (Roy & Ward, 1990).

Identification of the species was based on the fermentation patterns of the various isolates using the API 50CHL system.

RESULTS AND DISCUSSION

The culture from Chr Hansen's Laboratories was used to choose the best medium for growth and enumeration, and the final selection(s) was established by comparing the recoveries in (a) the non-selective and (b) the selective media.

It was found that, using the same pure culture of *Bifidobacterium bifidum* and the same amount of inoculum, the highest number of bifidobacteria was recovered by Rogosa's modified agar (Table 2). The appearance of the colonies varied in size and shape throughout the medium. Surface colonies were mainly convex, round and opaque, colonies below the surface

TABLE 2
Comparison of total colony counts on non-selective media

Medium (agar)	*Dilution plated* 10^{-7}	10^{-8}	10^{-9}
Rogosa modified*	162±11	15±1	2±1
Tomato juice*	115±17	12±4	1±1
Tryptone phytone yeast extract*	140±17	17±3	4±2
Blood liver†	54±5	7±2	0±0
Orla-Jensen†	68±2	6±2	0±0
MRS‡	49	5	1

*Figures are overall means (±SD) of four experiments.
†Figures are overall means (±SD) of two experiments.
‡Figures of one experiment.
(See text for details of media).

were lenticular and colonies at the bottom of the petri dish were large and rounded, or irregular in shape. Under the microscope, bifidobacteria appeared pleomorphic. Curved or straight rods, with or without 'bulbous' ends were numerous, but many organisms showed varying degrees of branching. Many bifurcated Y and V forms were observed, and occasionally branching was seen at both ends. In some instances the branching was so profound that any resemblance to rods was lost.

For the selective isolation of bifidobacteria, the agents shown in Table 1 were added to Rogosa's modified agar or TPY agar. The effectiveness of these substances depends mainly on the species, the number of bacteria, the kind and concentration of the selective agent and the growth medium (Kurmann & Rasic, 1983).

A comparison of the total counts of *Bifidobacterium bifidum* on Rogosa's (selective) and TPY (selective) media (see Table 3) and non-selective variants of the same media (see Table 2) confirmed that the selective agents did not depress the ability of the preferred media to support the growth of bifidobacteria.

TABLE 3

Comparison of total colony counts on two selective media; all figures are means (± standard deviations) from a minimum of four experiments

Dilution	*Rogosa's selective*	*Tryptone phytone yeast extract selective*
10^{7}	141±15	93±6
10^{8}	12±2	15±2
10^{9}	1±1	2±1

Bifidobacteria grown on a selective medium have more irregular shapes than on the same non-selective medium, and it may be that the presence of inhibitors (selective agents) causes alterations in morphology, so giving rise to more branched cells.

In order to check the selectivity of the media, a culture of *Bifidobacterium bifidum* was mixed with a yogurt culture (*L bulgaricus*:*S thermophilus* - 1:1) in the ratio of *B bifidum*:yogurt culture - 1:1. The best selective medium was that which gave the highest counts of *B bifidum* without any growth of lactobacilli or streptococci, and that was Rogosa's modified selective medium (4.0 ml PPNL/100 ml medium). TPY medium also gave high counts of *B bifidum*, and seems suitable for the selective isolation and growth of bifidobacteria.

For the isolation of *Bifidobacterium* sp from commercial products, Rogosa's modified selective agar was used. The assignment of a strain of commercial origin to the genus *Bifidobacterium* was based upon the detection of fructose-6-phosphate phosphoketolase (F6PPK) activity. With this test, an isolate from sample 2 (see Table 4) proved to belong to genus *Bifidobacterium*, whereas isolates from samples 1 and 3 gave negative results.

TABLE 4

Fermentation patterns of species isolated from commercial products

	Isolate from samples			*Bifidobacterium*			
*Sugar**	*2*	*1*	*3*	*adolescentis*	*longum*	*breve*	*bifidum*
Arabinose	+	+	–	+	+	–	–
Ribose	+	+	–	+	+	+	–
Xylose	–	+	–	+	d	–	–
Mannitol	–	–	–	d	–	d	–
Salicine	+	–	–	+	–	+	–
Cellobiose	+	–	–	+	–	d	–
Trehalose	+	–	–	d	–	d	–
Melizitoze	–	–	–	d	+	d	–

d = Differential reaction according to strain.

*After Scardovi (1986).

Identification:

Sample 1 – *Bifidobacterium adolescentis*
2 – *Bifidobacterium longum*
3 – *Bifidobacterium bifidum*

Further testing, using the API ZYM system, was carried out to confirm the above result for sample 2, and to establish whether or not the isolates from samples 1 and 3, that gave a negative result in the F6PPK test, belonged to the genus *Bifidobacterium*. Isolates from sample 2 and sample 3 gave the expected results, ie, positive to all three enzymes, whereas the isolate from sample 1 did not, even though it had the morphology of a typical *Bifidobacterium* sp. It was interesting that although the identity of the isolate seemed obvious, eg, from the morphology or from the survival in certain selective media, definite proof remained elusive.

After 'confirming' the genus, biochemical tests were performed to establish the fermentation patterns of the isolates. In particular, the utilization of L-arabinose, cellobiose and ribose was examined. The reason for selecting these three sugars is that they give a clear fermentation profile for the three/four species usually employed in the production of fermented milks. For example, a *Bifidobacterium* sp used in milk fermentation that gives an arabinose (+ve), cellobiose (+ve) and ribose (+ve) reaction, is most probably *B adolescentis* (Table 4).

From these results, it is likely that selective modified Rogosa's agar could be employed to monitor the survival of bifidobacteria in a range of dairy products, and in the presence of other lactic acid bacteria.

AS is grateful to Sanofi Bio-Industries, Paris, France, for the financial support that made it possible to complete this work, and to Pateras scholarship foundation, Pireus, Greece, for a most generous Postgraduate Scholarship.

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Some aspects of the production of a concentrated yoghurt (labneh) popular in the Middle East

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1. Introduction

The lactic acid fermentation is one of the oldest methods employed for the preservation of milk, but even these relatively acidic products can still be prone to spoilage. However, a further improvement in keeping quality can sometimes be achieved by concentration, and in some rural communities of the Middle East, concentrated yoghurt, produced from the surplus of summer milk, is essential as a foodstuff for the winter. There are various means by which fermented milks can be concentrated, and Table 1 indicates some of the traditional methods of processing, and the name by which the various products are known in different countries of the Middle East.

These condensed yoghurts play a major role in the diet of Middle Eastern communities, and "Labneh", for example, is often consumed, especially during the hot season, with bread as the major part of a meal. In addition, other types of condensed product can be manufactured from "Labneh" (see Figure 1), and hence impart a degree of variety to the diet. The production of these various foodstuffs froms an age-old tradition for many rural or nomadic groups, but, with the spread of urban development, attempts have been made to modify these traditional practices for factory-scale production.

2. Objective

This trend has brought with it demands for product uniformity, and sometimes a requirement that the material should conform to legal compositional standards. However, detailed knowledge of "Labneh" and its production is scarce, and hence it was the objective of this study to investigate on a laboratory-scale (using the traditional cloth-bag method) the influence of certain factors on the quality of concentrated yoghurt. The level of total solids in the original milk was selected as one feature of possible significance, while the effect of using various strains of yoghurt starter was also studied. The relationship between the level of total solids in the milk for yoghurt production and the rate of whey drainage during the process of concentration was also examined, not only because it can influcence the economics of the process, but also

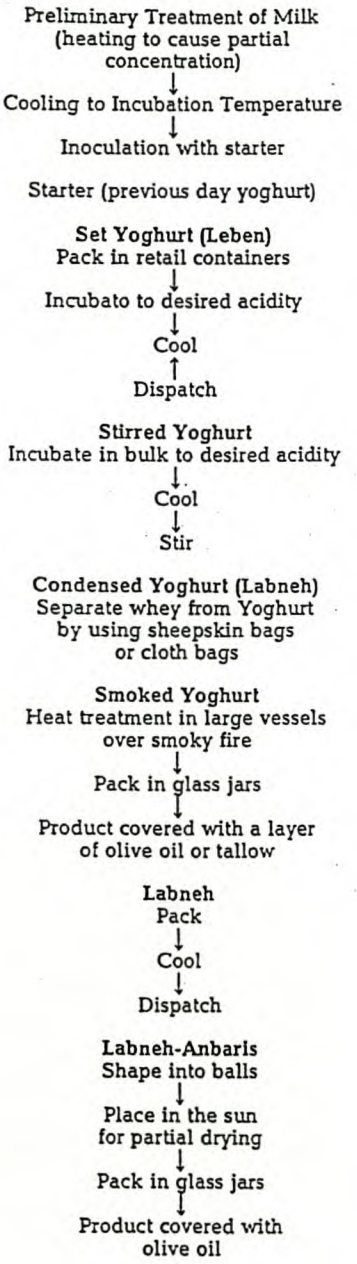
Table 1: The terminology of condensed yoghurt as used in the Middle East, and the means by which the original yoghurt is concentrated*)

Traditional Name	Country of Origin	Method of Concentration
Labneh or Lebneh	Lebanon and some Arab countries	Cloth bag
Tan or Than	Armenia	Cloth bag
Torba and Kurut	Turkey	Cloth bag
Tulum	Turkey	Goat or Sheep's Skin bag
Leben Zeer	Egypt	Earthenware Vessel

After HEINEMANN (1), YONEZ (2), VAYGIN (3), and MORCOS *et al.* (4).

*) The milk used for the initial fermentation can be either cow's, sheep's, goat's, buffalo's or a mixture. The concentration can be achieved either by producing the yoghurt in a hanging cloth bag so that the whey seeps out, or, to increase the rate of whey drainage, by piling the bags on top of each other to exert pressure.

Fig. 1: The production of yoghurt and related products traditionally made in the Middle East



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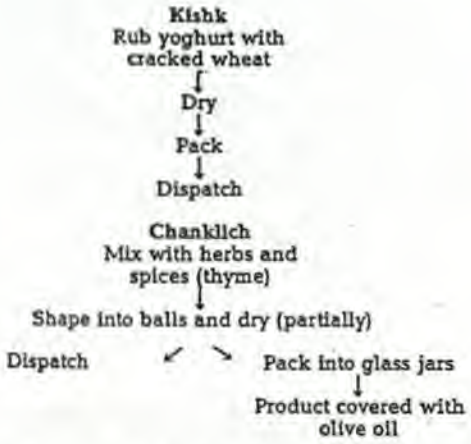

because it can substantially modify the consistency of the concentrated product. It was also apparent that objective criteria for assessing the quality of condensed yoghurt were not well established, and hence it became necessary, as an additional facet of the main study, to establish certain characteristics of the product and evaluate possible methods of appraisal.

3. Materials and Methods

Full cream spray-dried milk powder was used for the production of the natural yoghurt, and the concentrated yoghurt (Labneh) was derived from this. The laboratory process is illustrated in Figure 2.

Fig 2 Flow Diagram of Laboratory Scale Production of Yoghurt and Concentrated Yoghurt

¹) The cloth bag was made from a double layer of cheese cloth, and measured 33 x 38 cm when spread. A draw-string closure served to suspend the bag in the refrigerator.

The yoghurt cultures used in this study were mainly commerical starters, and consisted of the following organisms: *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The strains used were (CH-1) and (Boll-3) from Chr. Hansen's Laboratorium, Denmark, and a starter (coded RR) kindly supplied by Dr. Th. Galesloot, NIZO, Holland. These starters were obtained in a freeze-dried form, and the working stocks were maintained in sterile skim milk (9% TS), (5).

The routine appraisal of quality of the end product was confined to measurements of titratable acidity and consistency, but organoleptic considerations were, as far as was possible, also taken into account. However, the emergence of legal standards raises the additional question of chemical composition, and an analysis for total solids and fat was carried out for comparative purposes. The fat content was obtained using the Gerber method (6); for "Labneh", the method was modified slightly in that 5.15 g. of product were used, and the result was adjusted accordingly.

The consistency of the products were measured along the lines indicated by ROBINSON and TAMIME (7), as was the titratable acidity. However, with the "Labneh", the percentage lactic acid was determined using a 5 g. sample mixed with 5 ml. of warm, distilled water; the titre (X2) gave the result as % lactic acid in the original material. The level of total solids in the various samples was determined gravimetrically (British Standards, 8).

4. Results

In order to have some guidelines as to the nature of the commercially available product, three different brands of "Labneh" were transported from the Lebanon by air in a refrigerated container. The analysis of these samples is shown in Table 2.

Table 2: Analysis of some typical samples of commercial "Labneh" from the Lebanon

Sample	% Total Solids	% Fat	% Lactic acid	Penetrometer* Reading in (1/10 mm)
A	22.90	10.50	1.658	150
B	21.78	8.40	2.528	140
C	21.67	8.20	2.076	145
Standard	26.00	10.00	–	–

Although the Lebanese Standards (9) stipulate that "Labneh" must contain a minimum of 26% total solids, the commercial samples were, according to our results, rather below this figure. Nevertheless, when the samples were assessed organoleptically by a taste panel familiar with the product, acceptability was excellent, and sample (A) (1.6% lactic acid) was, in particular, rated highly. It was decided, therefore, to produce concentrated yoghurt of 22–23% total solids, i. e. a product giving a consistency reading of 140–150 with the penetrometer, and to use whole milk ranging from 12–20% TS as the starting material.

Some preliminary studies suggested that when natural yoghurt was assessed organoleptically (10), the highest score was attained by test samples with 16% TS. The transformation of this product into "Labneh" (24% TS) necessitated the removal of 1300 ml. of whey/3 kg. of original yoghurt, and this rate of extraction was applied subsequently to all the batches of yoghurt prepared (i. e. 12–20% TS). In this way, the influence of different total solids levels in the "Labneh" could be assessed, and also the effect of total solids (original yoghurt) on both the extraction rate and composition of the whey. An additional reason for adopting this procedure was that, if it is desirable that natural yoghurt should have 16% TS., then commercially it would be attractive if the 'Labneh" could be made by simply diverting a portion of the standard production material.

The results of this experiment are shown in Table 3 and it can be seen that a natural yoghurt (16% TS) gives rise to a concentrated product very similar to its

commercial counterpart, i. e. 1.7% lactic acid and penetrometer reading – 140. It was also established that, if the total solids in the "Labneh" fell below 20%, then the product was undesirably thin and tasteless, while above 25% TS, it was rather "gummy" and almost bitter in taste. The initial total solids also exerted a marked influence on the rate of whey drainage, and, as can be seen from Figure 3, a starting figure of 16% TS again gave the most desirable result. Thus, a potential drainage time of 14 hours means that concentration can be carried out overnight, and hence to a large degree, fit in with the normal working hours of a dairy.

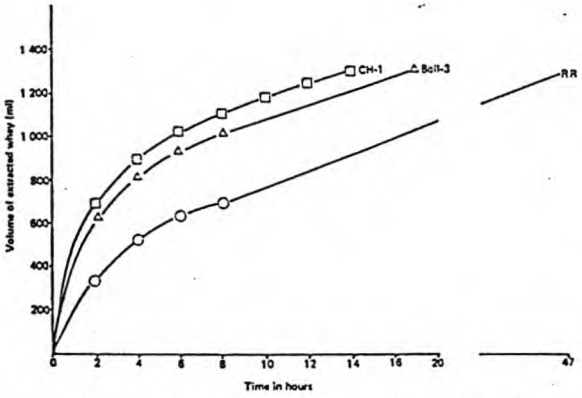

Fig. 3: Rate of whey drainage from yoghurt made with the levels of total solids indicated starter culture (CH-1)

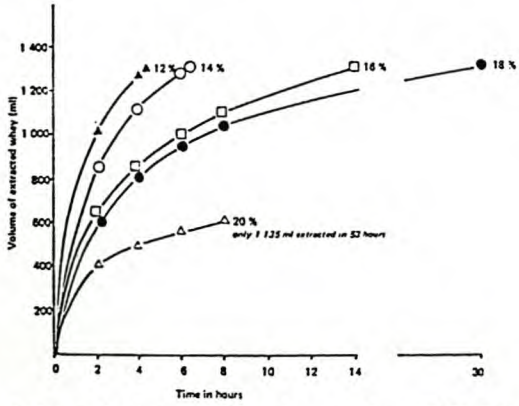

Fig. 4: Rate of whey drainage from yoghurt (16% TS) using different starter cultures

It can be concluded, therefore, that, employing the traditional method of concentration, 16% total solids represents the optimum starting mix for the production of "Labneh". Consequently, this level of solids was selected for the series of experiments designed to assesss the possible influence of the various starter cultures. The results are shown in Table 4, and, with the exception of consistency, the concentrated products were broadly similar. However, the "Labneh" made with starter (RR) was much too viscous for normal acceptability, while the whey extraction time to reach around 24% TS was 47 hours (see Figure 4), as against 14 hours for starter (CH-1). This

Table 3: Analysis of "Labneh" made from yoghurt with different levels of total solids*

%TS in Yoghurt Milk	% TS in Whey	% TS in "Labneh"	% Lactic Acid	Penetrometer Reading in 1/10 mm
12	5.72	17.66	1.446	277.5
14	6.97	20.88	1.644	197
16	8.21	23.44	1.768	140
18	9.38	25.13	1.982	117
20	10.63	28.29**	2.096	84

* Starter used was (CH-1)
** Only 1135 ml extracted

Table 4 Analysis of "Labneh" made from 16% TS yoghurt mix and using three strains of starter culture

Type of Starter used	% of TS in Whey	% TS in "Labneh"	% Lactic acid	Penetrometer Reading in 1/10 mm
CH-1	8.21	23.44	1.768	140
Boll-3	7.96	23.70	1.802	125
RR	7.27	23.97	1.916	110

resistance to whey loss appears to stem from the secretion, by the constituent organisms of culture (RR), of a colloidal material capable of water retention. The nature of this secretion was not known, but the work of SHARPE *et al* (11) with other lactic acid bacteria suggested that it might be a carbohydrate polymer. Consequently, whey, collected from yoghurt made with starter (RR), was examined for the presence of an hydrocolloidal polysaccharide. The technique employed was a modification of the method of CLARKE & STONE (12), with ethanol being employed as the precipitant in place of ammonium sulphate (13). After purification, the material, which was readily soluble in warm water to give a viscous solution, was hydrolysed with N H_2SO_4. The hydrolysate was then neutralised and examined chromatographically (14, 15). The only monosaccharide recovered was glucose, and it was concluded that the polysaccharide secreted by starter culture (6RR) could well bea "glucan". Comparative analysis against a known sample of β-glucan confirmed this view. A more detailed chemical examination of the polysaccharide is obviously in order, but the present information provides an adequate explanation both of the influence of starter (RR) on the consistency of the "Labneh", and of the ability of the original yoghurt coagulum to retain whey during the process of extraction.

5. Discussion

The observed preference for natural yoghurt with a total solids level of 16% is not unexpected (16), but it may be relevant that the same material forms an excellent starting point for the production of concentrated yoghurt (24% TS). Thus, when the traditional process was employed, the "Labneh" was awarded an extremely high score, and was comparable with the best of the commercial brands. However, it was curious that none of the acceptable test products was able to meet the legal standard for concentrated yoghurt laid down in the Lebanon. It was also apparent that the standard could not be attained without a considerable manipulation of the process (a view presumably shared by the commercial companies), and it is pertinent to question whether, in fact, the specification should not be reexamined.

The fact that normal commercial starter cultures can give rise to an acceptable concentrated product is to be expected, but the behaviour of culture (RR) deserves comment. Thus, the improvement in viscosity of both the natural yoghurt and the "Labneh" brought about by the presence of the "glucan" material could be relevant in two contexts. In the first place, manufacturers of stirred, fruit yoghurts could find that the polysaccharide could reduce the demand for stabilisers, while secondly, it is possible that concentrated yoghurt of acceptable consistency could be produced with lower levels of milk solids. The legal position is obviously relevant in this latter situation, but the economic advantages to the manufacturer could be important. This last point could be especially valid as mechanical methods of whey extraction come into operation (17), and closer control of the drainage process becomes possible. Obviously consumer reaction will need to be considered but the way is clearly open for some fundamental changes in the production of concentrated yoghurt.

Acknowledgement

The authors would like to thank Mr. D. T. Bourne, Arthur Guinness Son & Company Ltd., for providing them with a sample of β-glucan. One of us (A. Y. T.) is indebted to the British Council for financial support.

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7. Zusammenfassung

TAMIME, A. Y., ROBINSON, R. K.: Einige Gesichtspunkte der Herstellung eines im Mittleren Osten beliebten konzentrierten Joghurts (Labneh). Milchwissenschaft 33. (4) 209–212 (1978).

61 Joghurt (konzentriert).

Konzentrierter Joghurt hat viele Jahre in der Ernährung im Mittleren Osten eine Rolle gespielt. Die Herstellung blieb jedoch auf den privaten Haushalt beschränkt. Nunmehr ist ein Produkt, »Labneh«, im Handel, dessen Herstellung und Eigenschaften in der vorliegenden Arbeit untersucht werden.

Der angenehmste »Labneh« hatte eine Gesamttrockenmasse von 22 bis 23% und einen Säuregrad von 1,6–1,7% Milchsäure. Das optimale Ausgangsmaterial war natürlicher Joghurt von 16% Trockenmasse und bei Verwendung des traditionellen Herstellungsverfahrens konnten Abscheidungsgrad und Zusammensetzung der Molke standardisiert werden. Der Einfluß der Säurewecker erwies sich als wichtig. Ein Säurewecker war ungeeignet, da er beim Wachstum »Glucan« produzierte; die Wirkung dieses Polysaccharids machte es fast unmöglich, den gewünschten Molkenabflußgrad zu erreichen.

Die Eigenschaften des konzentrierten Produktes werden auch bezüglich rechtlicher Vorschriften und zukünftiger Herstellungsverfahren diskutiert. Dok.-Ref.

TAMIME, A. Y., ROBINSON, R. K.: Some aspects of the production of a concentrated yoghurt (Labneh) popular in the Middle East. Milchwissenschaft 33. (4) 209–212 (1978).

61 Yoghurt (concentrated).

Conentrated yoghurts have for many years played an important role in the diet of Middle Eastern Communities, but production has always been on a domestic-scale. However, one product, Labneh, is now available commercially, and it is the manufacture and nature of this material that forms the subject of this study.

In particular it was shown that the most acceptable "Labneh" had a level of total solids (TS) of 22–23% and an acidity of 1.6–1.7% lactic acid. The optimum starting material for the process was natural yoghurt of 16% TS, and, using the traditional method of production, both the rate of extraction and the composition of the whey could be standardised. The influence of starter cultures was also shown to be important, and one starter was expecially unsuitable as it produced a "glucan" material during growth; the effect of this polysaccharide was to make achievement of the desired degree of whey drainage almost impossible.

The characteristics of the concentrated product are also discussed in relation to proposed legal standards, and possible future developments in processing are considered.

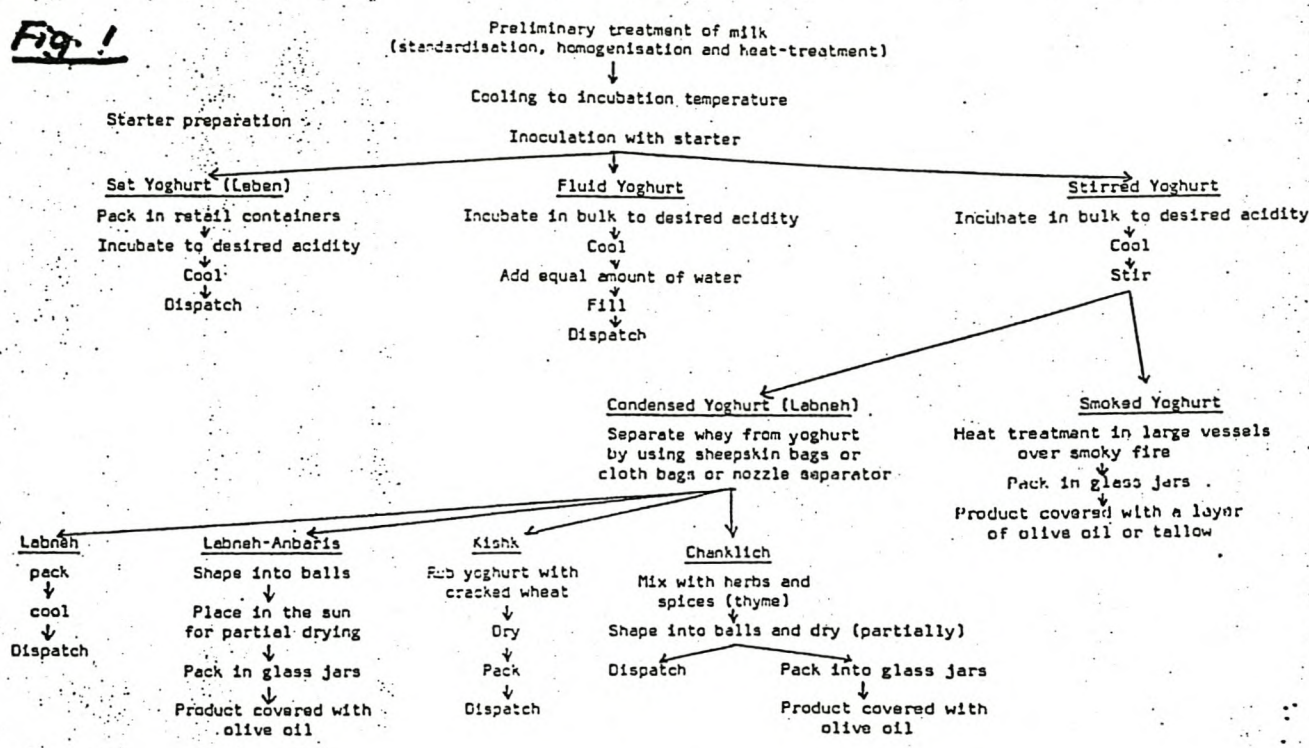
TAMIME, A. Y., ROBINSON, R. K.: Quelques aspects de la production d'un jaourt concentré (Labneh) populaire au Moyen Orient. Milchwissenschaft 33. (4) 209–212 (1978).

61 Jaourt (concentré).

TAMIME, A. Y., ROBINSON, R. K.: Unos aspectos de la producción de un yogur concentrado (Labneh) popular en el Oriente Medio. Milchwissenschaft 33. (4) 209–212 (1978).

61 Yogur (concentrado).

Fig. 1



A DAIRY PRODUCT FOR THE FUTURE — CONCENTRATED YOGHOURT.

by

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ABSTRACT

Concentrated yoghourts have for many years played an important role in the diet of certain populations, but production has always been on a domestic scale. However, similar products are now available commercially, and it is the manufacture and nature of these materials that forms the subject of this study.

In particular it was shown that the most acceptable 'yoghourt' had a level of total solids (T.S) of 22 — 23% and an acidity of 1.6 — 1.7% lactic acid. The optimum starting material for the process was natural yoghourt of 16% TS, and, using the traditional method of production, both the rate of extraction and the composition of the whey could be standardised.

The characteristics of the concentrated product are also discussed in relation to proposed legal standards, and possible future developments in processing are mentioned.

It was reported by Doidge (1975) that the Bantu peoples form a low-consumption group in respect to cheese, and yet, in climatically similar areas in North Africa, fermented dairy products figure prominently in the diet. The reasons for this difference are obviously complex, but one aspect may simply involve taste. Thus, the bland flavour of products oriented towards European markets is quite often unacceptable to other groups, and the question arises as to whether the manufacture of 'special brand' commodities may not provide a means of ending the low level of consumer interest.

The feasibility of this idea is clearly demonstrated in relation to an 'amenable' product like yoghourt, and, in the UK for example, natural set yoghourts produced to an exceptionally high viscosity and acidity are rapidly finding favour with certain ethnic groups. An alternative approach to meeting this demand would be to produce concentrated yoghourt, in which natural yoghourt is created by removing some of the whey. The advantage of this process is that the end-product is vastly superior, in terms of flavour and consistency, to a normal yoghourt of equivalent total solids. The exact reasons for this difference are not known, and even details of the procedure for making condensed yoghourt are scarce. Nevertheless, the concentration process does offer the prospect of products with a distinctive consumer appeal, and hence it was decided that an investigation, on a laboratory-scale, of the influence of certain factors on the quality of concentrated yoghourt might prove to be of interest.

The relationship between the level of total solids in the milk for yoghourt production and the rate of whey drainage during the process of concentration was selected as one feature of possible significance, as was the associated question of the optimum level of total solids for the retail product. It was also apparent that objective criteria for assessing the quality of condensed yoghourt were not well established, and hence, it became necessary, as an additional facet of the main study, to establish certain characteristics of the product and to evaluate possible methods of appraisal.

Material and Methods

Full-cream spray-dried milk powder was used for the production of natural yoghourt, and the concentrated yoghourt was derived from this. The laboratory process is illustrated in Figure 1. The starter culture employed in this study was of commercial origin

FIGURE 1. Flow diagram of laboratory scale production of Yoghourt and Concentrated Yoghourt

** The cloth bag was made from a double layer of cheese cloth, and measured 33 x 38cm when spread. A draw-string closure served to suspend the bag in the refrigerator.*

TABLE 1. Analysis of some typical samples of commercially available concentrated yoghourt from the Lebanon.

Sample	% Total solids	% Fat	% Lactic acid	Penetrometer *Reading (1/10mm)*
A	22.90	10.50	1.658	150
B	21.78	8.40	2.528	140
C	21.67	8.20	2.076	145
Lebanese Standard	26.00	10.00		—

(CH-1, Chr. Hansen's Laboratorium, Denmark), and consisted of a balanced mixture of *Streptococcus thermophilus* and *Lactobacillus bulgarius*. The original culture was obtained in a freeze-dried form, and the working stocks were maintained in sterile skim-milk (9% TS), (Tamime & Robinson, 1976).

The routine appraisal of quality of the end product was confined to measurements of titratable acidity and consistency, but organoleptic considerations were, as far as was possible, also taken into account. However, the emergence of legal standards raises the additional question of chemical composition, and an analysis for total solids and fat was carried out for comparative purposes. The fat content was obtained using the Gerber method (Pearson, 1970) with the method modified slightly in that 5.65 g of the concentrated product were used, and the result adjusted accordingly.

The consistency of the products was measured along the lines indicated by Robinson and Tamime (1976), as was the titratable acidity, except that the percentage lactic acid of the condensed yoghourt was determined using a 5 g sample mixed with 5 ml of warm, distilled water; the titre (x2) gave the result as % lactic acid in the original material. The level of total solids in the various samples was determined gravimetrically (British Standards, 1963).

Results

In order to have some guidelines as to the nature of the commercially available product, three different brands of concentrated yoghourt were transported from the Lebanon by air in a refrigerated container. The analysis of these samples is shown in Table 1.

Although the Lebanese Standards (1965) stipulate that the product must contain a minimum of 26% total solids, the commercial samples were, according to our results, rather below this figure. Nevertheless, when the samples were assessed organoleptically by a taste panel familiar with the product, acceptability was excellent, and sample (A) (1.6% lactic acid) was, in particular, rated highly. It was decided, therefore, to produce concentrated yoghourt of 22-23% total solids, i.e. a product giving a consistency reading of 140-150 with the penetrometer, and to use whole milk ranging from 12-20% TS as the starting material.

Some preliminary studies suggested that when natural yoghourt was assessed organoleptically (Pearce and Heap, 1974), the highest score was attained by test samples with 16% TS. The transformation of this product into the condensed variety (assumed 24% TS) necessitated the removal of 1300 ml of whey/3 kg of original yoghourt, and this rate of extraction was applied subsequently to all the batches of yoghourt prepared (i.e. 12-20% TS). In this way, the influence of different total solids levels in the concentrated material could be assessed, and also the effect of total solids (original yoghourt) on both the extraction rate and composition of the whey. An additional reason for adopting this procedure was that, if it is desirable that natural yoghourt should have 16% TS., then commercially, it would be attractive if condensed yoghourt could be made by simply diverting a portion of the standard production material.

The results of this experiment are shown in Table 2 and it can be seen that a natural yoghourt (16% TS) gives rise to a concentrated product very similar to the favoured commercial counterpart, i.e. 1.7% lactic acid and penetrometer reading — 140. It was also established that, if the total solids in the end-product fell below 20%, then the material was undesirably 'thin and tasteless', while above 25% TS., it was rather 'gummy' and almost bitter in taste. The initial total solids also exerted a marked influence on the rate of whey drainage, and, as can be seen from Figure 2, a starting figure of 16% TS again gave the most desirable result. Thus, a potential drainage time of 14 hours means that concentration can be carried out overnight,

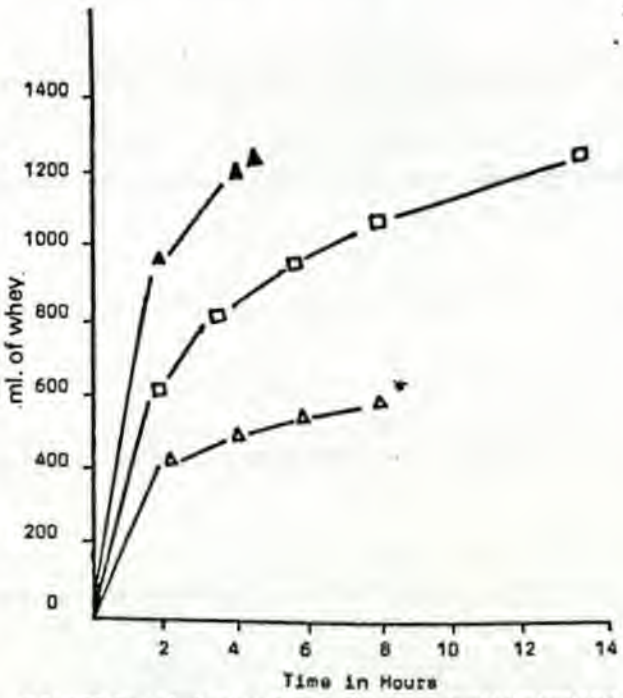

FIGURE 2. Rate of whey loss from yoghourts made with the levels of total solids indicated. (Tamime, A.Y. 1977)
▲ 12% □16% △ 20%*only 1135ml extracted in 53 hours.

TABLE 2. *Analysis of concentrated yoghourt made from Yoghourt with Different levels of Total Solids*

% TS in Yoghourt Milk	% TS in Whey	% TS in Yoghourt (Conc.)	% Lactic Acid	Penetrometer *Reading (1/10mm)*
12	5.72	17.66	1.446	277.5
14	6.97	20.88	1.644	197
16	8.21	23.44	1.768	140
18	9.38	25.13	1.982	117
20	10.63	28.29*	2.096	84

* Only 1135 ml extracted

and hence to a large degree, fit in with the normal working hours of a dairy.

It could be concluded, therefore, that employing the traditional method of concentration, 16% total solids represents the optimum starting mix for the production of concentrated yoghourt, and that the end-product from such a process could be comparable with the best of the currently available commercial brands. However, it was curious that none of the routine test products would have been able to meet, without considerable ingredient manipulation, the emerging legal standards for condensed yoghourt, and it is clearly possible that the specifications will have to be re-examined.

Discussion

The observed preference for natural yoghourt with a total solids level of 16% is not unexpected (Robinson & Tamine, 1975), but it may be relevant that the same material forms an excellent starting point for the production of concentrated yoghourt (23% TS). The creamy consistency of this latter product, together with its sharp, aromatic flavour, identifies it as a material very different from a normal yoghourt, and one which could have considerable consumer appeal.

The entrenched preference of Europeans for fruit/flavoured yoghourts may well prove a limiting factor in some markets, but, in other areas, the reaction could be markedly different. It is clearly possible, therefore, that concentrated yoghourt could be a product for future development, particularly as the process of bulk concentration can now be carried out mechanically (Norling, 1976). Consumer reaction will obviously be the ultimate judge, but the way is now clearly open for some fundamental changes in the nature of 'yoghourt.'

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RHEOLOGICAL PROPERTIES OF CONCENTRATED YOGHURT (LABNEH)

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ABSTRACT

The effect of six techniques for the elevation of the total solids on the rheological properties of labneh were investigated using controlled-stress dynamic rheometer. All samples exhibited a weak viscoelastic gel structure with the storage modulus (G') higher than the loss modulus (G") over all of the measured range. None of the experimental materials produced the same overall gel strength as the control made by draining with traditional cloth-bags. The changes in the storage modulus (G') as a function of amplitude sweep were mirrored by changes in the loss modulus (G"). Considerable differences in the loss tangent (G"/G') values of the various materials were observed at higher stress amplitudes. Rheological differences in the overall gel strength at low amplitude and frequency suggest that, although the type of protein interactions in each case may be similar, there are differences in the degree of interactions. Subsequent breakdown at higher amplitudes and frequencies suggests that the overall domains of the treated proteins may have been reduced, and that different methods of manufacture may be producing materials that have different space occupancy in the gel.

INTRODUCTION

Labneh (a concentrated fermented milk product) has increased in popularity during recent years. Its increasing economic importance has been achieved as a result of its perceived nutritional benefits and storage characteristics (Benezech

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and Maingonnat 1994). However, much of its consumer acceptability is dependent on its sensory properties which, in turn, seem to be heavily dependent on the method of processing of the material. It should be possible to use rheological data to modify and control the process conditions to give a product with the quality attributes desired by the consumer. Over the last 10–15 years, conventional methods such as the Posthumus funnel (Posthumus 1954), the Plummet device (Tamime and Robinson 1985) and the falling ball apparatus (Bottazi 1976) have been almost universally accepted for the measurement of physical properties of stirred yoghurt, and rotational viscometers, such as the Brookfield (Abrahamsen and Holmen 1980) and the Haake (Parnell-Cluines *et al.* 1986) viscometers, have also become widely used.

For set yoghurt, the firmness of the body/gel is considered to be the best parameter to assess the physical quality of the system. The consistometer or penetrometer apparatus have been widely used for this purpose. The penetrometer test measures the force required to push a probe into a food or depth of penetration but, as a wide variety of probes, penetration depths and temperatures may be used, it is almost impossible to compare results between laboratories. In order to standardize the results and to improve the reproducibility of the data derived from penetrometer-type measurements, a computerized Texture Profile Analyzer (TPA) is usually employed to study the texture of set-type yoghurts (Benezech and Maingonnat 1994). All of the techniques mentioned above have some advantages and disadvantages. Yoghurt is defined as a weak gel system and weak gels are unable to keep their structural integrities during high shear; even the lowest possible shear may disturb the gel when traditional penetrometer or rotational techniques are used. Dynamic oscillatory testing is a technique that is very appropriate for investigating the structure of a viscoelastic material such as yoghurt (Steventon *et al.* 1990; Xiong and Kinsella 1991).

Several studies have investigated the viscoelastic properties of yoghurt-type materials; (1) the effect of starter culture (Rohm and Kovac 1994); (2) different milk types (Biliaderis *et al.* 1992; Vlahapoulou and Bell 1993); (3) rheological characteristics of stirred yoghurt (Skriver *et al.* 1993; Rohm 1992; Ramaswany and Basak 1991, 1992). Horne (1993) found that these dynamic measurements showed good correlation with certain rheological measurements of gel strength (elastic modulus) in set yoghurts. In spite of the fact that rheological characterization of normal set and stirred yoghurts have been investigated by dynamic testing, there has been no study of the rheological properties of labneh. Labneh is traditionally manufactured using a cheese cloth bag for concentrating yoghurt after overnight refrigeration. Because it needs a long processing time (2–3 days), is labour-intensive and unhygienic, this method of manufacture is not suitable for large scale processing (Tamime *et al.* 1989). During the last decade, mechanized processes such as ultrafiltration (UF) and reverse osmosis (RO) have become wide-

ly used in the production of labneh. Especially, the concentration of warm yoghurt to the desired total solids level by UF after fermentation, is being currently practised intensively. So far, all mentioned manufacturing techniques have been studied individually and because different rheological techniques have been used in each study, it is difficult to reach a fair judgement about the physical qualities of the labnehs. The present study was designed to compare different manufacturing techniques in terms of physical characteristics of resulting products brought about by application of those techniques.

EXPERIMENTAL

Materials

Full cream milk powder supplied from Adams Food Ingredients Ltd. (Staff. UK) and stored at 4C was used. A freeze-dried commercial yoghurt culture (coded CH-1) from Chr. Hansen's Laboratory (Reading, UK) was used to prepare the yoghurt. The starter was a blend of *Streptococcus thermophilus* and *Lactobacillus delbruckii* sub-sp *bulgaricus* in equal proportions. The labneh samples were stored overnight at 4C before testing.

Methods

Yoghurt-making Process. Six different techniques for raising the level of total solids were applied: (1) traditional cloth bag system (control)-drainage was achieved by holding yoghurt (16% w/v solids) in bags of double layer cheese cloth at 4C for 18–20 h, (2) direct reconstitution of full-cream milk powder to 23% (w/v) total solids (direct reconstitution labneh), (3) reverse osmosis (RO) after and before fermentation, and (4) ultrafiltration (UF) after and before fermentation. Pilot scale UF and RO plants were employed for the concentration of milk and yoghurt to 23% total solids. The UF cartridge consisted of a bundle of tubular membranes and the specifications of the UF membranes were: surface area 0.8 m^2, type ES 625 (Patterson Candy, Whitchurch, Hands., UK), membranes composed of polyether sulphone, and nominal molecular weight cut-off 25,000 dalton; and the RO membranes were: surface area 1.2 m^2, type ZF 99 (Patterson Candy International), membrane composed of polyether sulphone. For the membrane processes, the temperatures of milk and yoghurt were 50C and 42C, respectively; the RO plant was operated at 20 bar for yoghurt and 25 bar for milk systems. For all samples, a standard yoghurt making procedure proposed by Tamime and Robinson (1985) was followed. The initial total solids content of the control, UF-after fermentation and RO-after fermentation labnehs was 16% (w/v), and the final total solids content for all end products was ~23%. The standard heat treat-

ment was 85C for 20 min and, after cooling, the milks were inoculated with a liquid starter culture (2% v/v) at 42C. Incubation was halted when the pH dropped to 4.3 for samples concentrated post-incubation, and 4.0 for previously concentrated samples; the final pH of all samples was 4.0. In the determination of the effect of the level of protein on the gel properties of labneh, milks were concentrated with UF up to 10% protein level. Then, serial dilutions from 1 to 9% protein content were made using the same permeate in order to keep the ionic environment identical in each sample.

Chemical Analyses. Chemical analyses were done according to the methods outlined in the British Standard Institution as follows: protein, BSI Part 10:1 (Kjeldahl N × 6.38) (BSI 1988); fat, Gerber method BSI 696: Part 2 (BSI 1989) and ash, BSI 1741: Part 9 (BSI 1988).

Dynamic Rheological Measurements. Dynamic rheological tests were performed with a Rheotech International controlled-stress oscillator rheometer using parallel plate geometry (10 mm plate radius and 1 mm gap setting). All samples were stirred by means of a controlled-speed stirrer at low speed before measurement in order to standardize between set and stirred labnehs. Each sample was loaded into the rheometer and allowed to relax and equilibrate to measuring temperature (2 min, 25C) prior to testing. A strain sweep used angular frequency, ω, from 10^{-3} to 10^{1} Hz at 0.07 mNm torque and sweeping amplitude from 1.5×10^{-2} to 1.5×10^{-1} mNm at 0.25 Hz. The rheometer was thermostatted by a water circulator connected to a temperature controller (Haake, UK). Measurements are the average of five replications and from each replication ten readings were taken. Standard errors are less than ±8% of average values for each sample.

Statistical Analyses. Differences between the samples were tested by single factor variation analysis using the data analysis tool kit that is supplied with Microsoft® Excel spreedsheet Version 5.0. Statistical differences between the groups were determined by DUNCAN test.

RESULTS AND DISCUSSIONS

Chemical composition of labnehs are illustrated in Table 1. In the RO and Direct reconstitution labnehs, the protein and fat contents increased relatively less than in the control and UF treated samples. The lactose concentration was reduced to 4.53 to 4.16% in the control and UF samples, whereas in the RO and direct reconstitution labnehs, this component increased with the concentration factor.

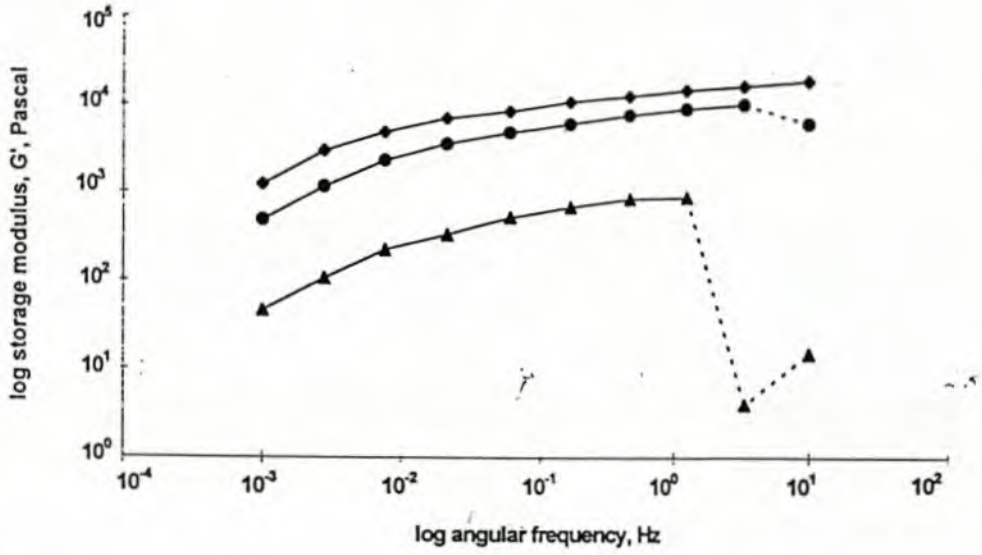
TABLE 1.
CHEMICAL COMPOSITION OF LABNEHS TESTED

Samples	Total solids %	Protein %	Lactose * %	Fat %	Ash %
Control labneh	23.31	9.20	4.16	9.18	0.79
UF after fermentation labneh	22.64	8.80	4.53	8.45	0.86
RO after fermentation labneh	22.22	6.38	8.24	6.60	1.00
UF before fermentation labneh	22.24	9.00	4.26	8.20	0.78
RO before fermentation labneh	23.22	6.82	9.07	6.25	1.08
Direct reconstitution labneh.	22.50	6.38	8.72	6.10	1.30

(*) Lactose was determined by difference.

The rheological analyses showed the labneh was a typical weak viscoelastic gel whose storage modulus (G′) was greater than loss modulus (G″) over the measured range and showing a frequency-dependence. None of the samples produced the same gel properties as the control sample, in spite of the fact that UF-treated samples (after and before fermentation) had approximately the same chemical composition as the control. A statistically significant difference was found between the samples ($P < 0.05$). Overall, the control was the stiffest sample followed by UF treated samples. RO and direct reconstitution applications created weaker structures in the final gels than control and UF treated ones. A structural breakdown was observed in all samples, with the exception of the control, at different point of frequency sweep (Fig. 1a and b). No slipping was observed in the measuring system. In general both network moduli increased with frequency in the linear region and higher protein content samples had higher storage and loss moduli. These profiles are consistent with the behaviour of weak viscoelastic gels (Ferry 1980; Benezech and Maingonnat 1994). Inhomogeneous particulate gels are generally more frequency-dependent than their homogeneous counterparts (Stading 1993), *i.e.*, the distribution of gel-forming components in the aqueous phase influences the frequency dependency of the gel. In the present case, a considerable frequency-dependency in all labnehs was evident. Therefore, it is justified to assume that the labnehs have an inhomogeneous particulate structure. The increase of network moduli with frequency, ω, indicates a relaxation of protein bonds during the period of measurements. Storage modulus (G′) is determined by the number and/or strength of nonrelaxing protein bonds, whereas loss modulus is determined by rapidly relaxing bonds (Roefs 1986). In the present case, until gel structure was broken down, the storage modulus was always higher than the loss modulus in all samples indicating that nonrelaxing bonds

(a)



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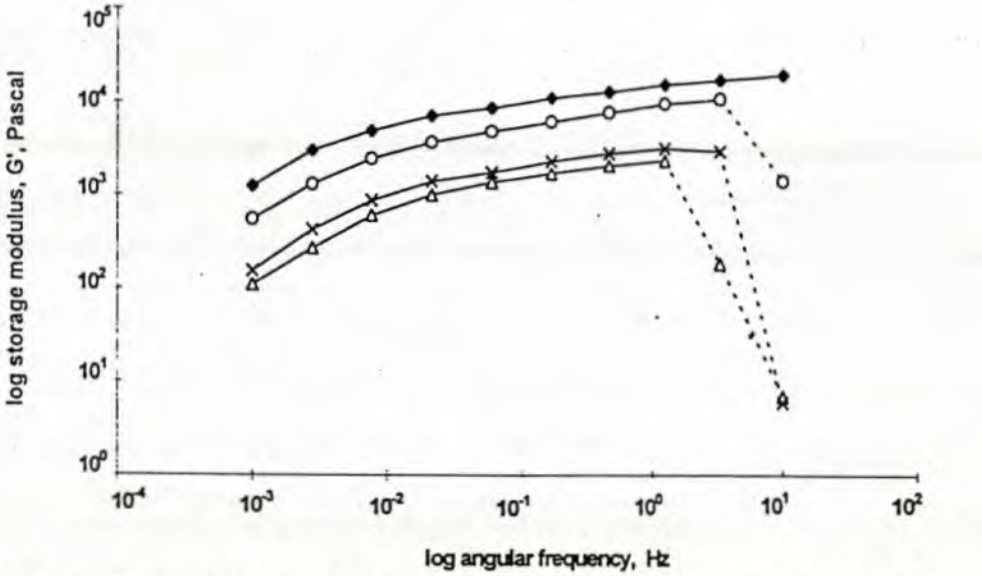
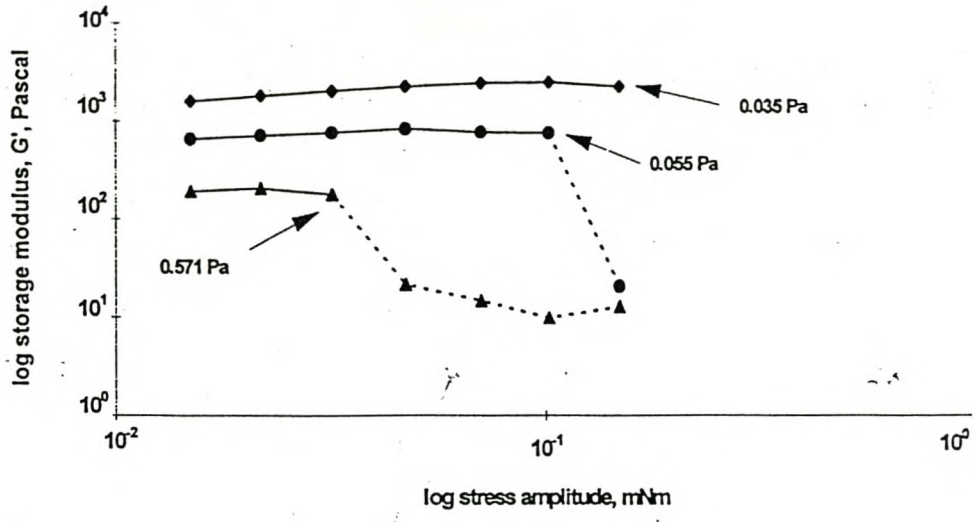


FIG. 1. STORAGE MODULUS OF SAMPLES CONCENTRATED (a) POST-FERMENTATION, (b) PRIOR TO FERMENTATION AS A FUNCTION OF ANGULAR FREQUENCY

Standard deviations are less than symbol dimensions. (♦) control, (▲) RO-after fermentation labneh, (●) UF-after fermentation labneh. (△) RO-before fermentation labneh, (○) UF-before fermentation labneh, (×) Direct reconstitution labneh.

(a)



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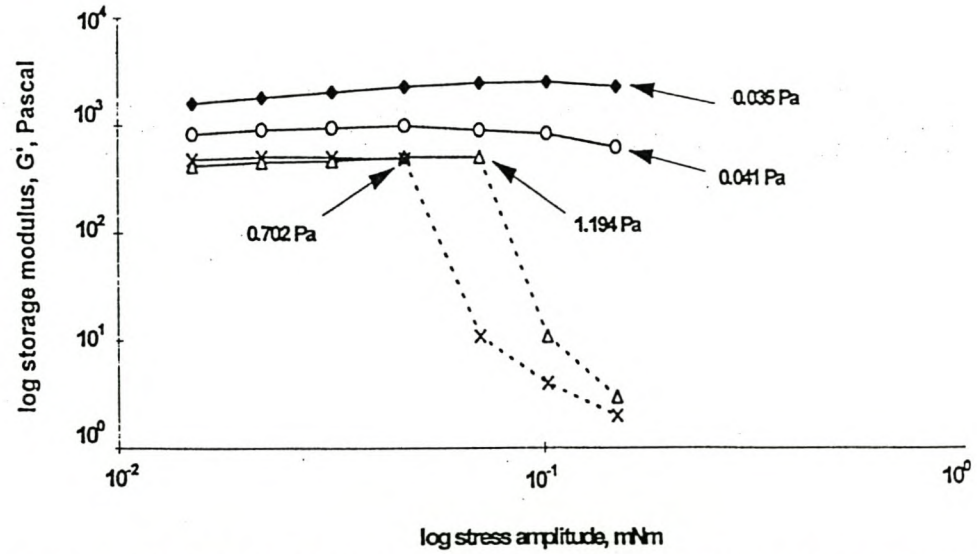

FIG. 2. STORAGE MODULUS OF SAMPLES CONCENTRATED (a) POST-FERMENTATION, (b) PRIOR TO FERMENTATION AS A FUNCTION OF AMPLITUDE SWEEP

Standard deviations are less than symbol dimensions. (♦) control, (▲) RO-after fermentation labneh, (●) UF-after fermentation labneh, △) RO-before fermentation labneh, (○) UF-before fermentation labneh, (÷) Direct reconstitution labneh. Points indicated give the amplitude strain at that stress.

dominated over rapidly breaking and reforming weak bonds, and this domination depends upon macromolecule (casein) concentration because the higher protein content samples had the higher storage moduli.

A linear viscoelastic region was evident for all samples. With the exception of the traditional and UF-before fermentation labnehs, a structural breakdown was observed at some point of the amplitude applied. Data for comparison purposes were also collected in the form of amplitude sweeps which were considered to be another indicative parameter of the structure. The control, UF-before and, to some extent, UF-after fermentation labnehs kept their structural integrities against increasing shear (Fig. 2a and b). However, the rest of the samples broke down at some point within the range of the amplitude applied. Corresponding strain amplitude data as a function of stress amplitude applied are presented in Table 2. The changes in the storage modulus (G′) as a function of amplitude were mirrored by changes in the loss modulus (G″) (Fig. 3a and b). This gave rise to considerable differences in the phase angle (tan δ) values of the various materials, especially at higher amplitudes (Fig. 4a and b).

Rheological properties of the control sample should be evaluated separately from the others, because physical characteristics of this sample came into being after fermentation was halted. However, in the other samples, rheological

TABLE 2.
STRAIN AMPLITUDE DATA OF LABNEHS AS A FUNCTION OF STRESS

	Strain amplitude (Pascal)					
Stress Amplitude mNm	Control labneh	UF after fer.labneh	RO after fer. labneh	UF before fer.labneh	RO before fer. labneh	Direct reconstitution labneh
0.015	0.006	0.015	0.055	0.010	0.021	0.021
0.022	0.007	0.019	0.101	0.013	0.027	0.027
0.032	0.009	0.025	0.571*	0.017	0.039	0.040
0.047	0.012	0.029	1.302	0.021	0.702*	0.054
0.070	0.016	0.037	3.251	0.024	1.053	1.194*
0.102	0.029	0.055	6.137	0.032	2.262	2.757
0.150	0.035	0.977*	12.416	0.041	4.354	5.604

(1) Stars indicate the strain at the gel breaking point.
fer. : fermentation

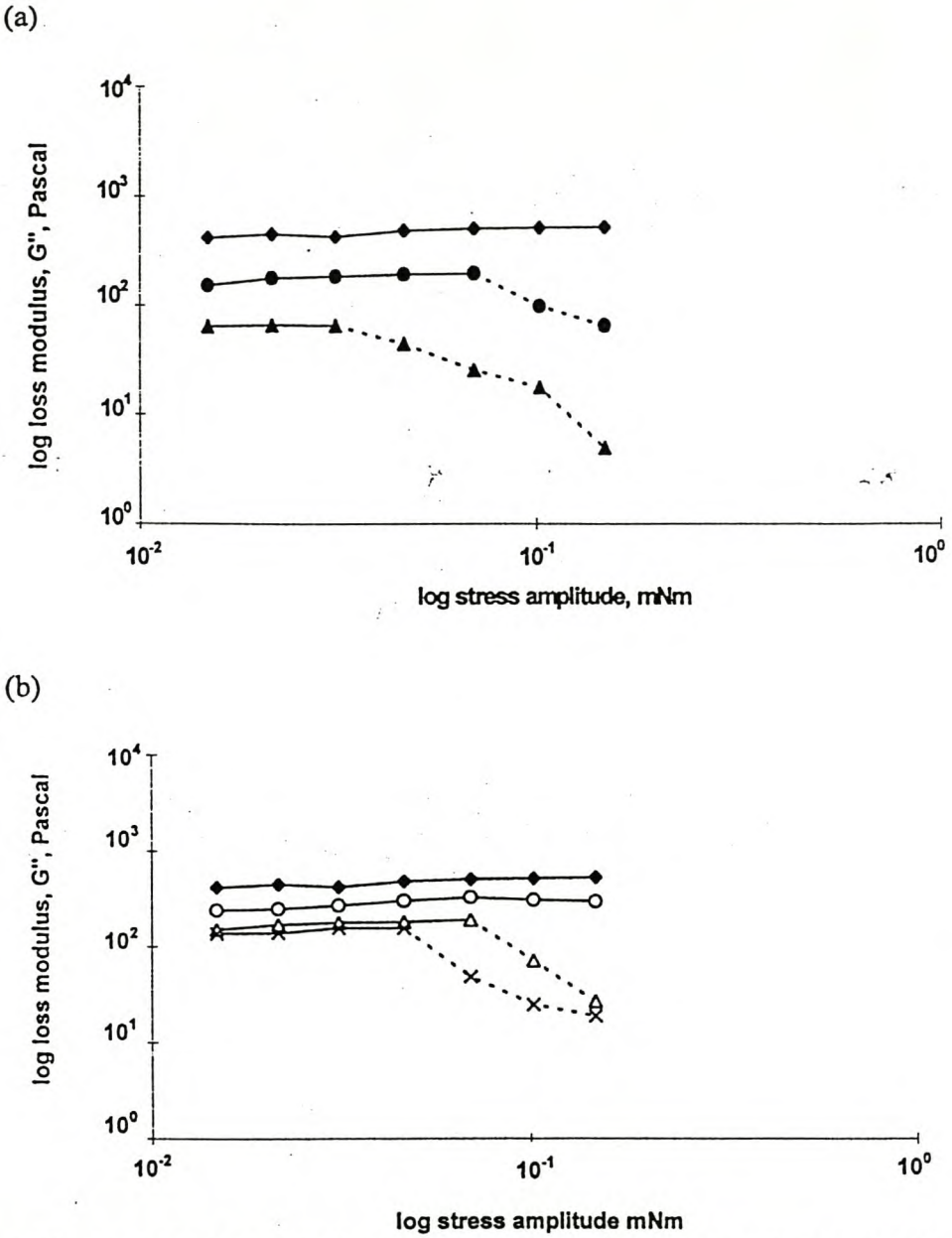

FIG. 3. LOSS MODULUS OF SAMPLES CONCENTRATED (a) POST-FERMENTATION, (b) PRIOR TO FERMENTATION AS A FUNCTION OF AMPLITUDE SWEEP

Standard deviations are less than symbol dimensions. (♦) control, (▲) RO-after fermentation labneh, (●) UF-after fermentation labneh, (△) RO-before fermentation labneh, (○) UF-before fermentation labneh, (×) Direct reconstitution labneh.

characteristics were determined during the fermentation stage. UF-after and RO-after fermentation labnehs were produced after the fermentation stage had finished but, because the samples were not cooled down after fermentation and membrane techniques were applied at incubation temperature, it may be reasonable to assume

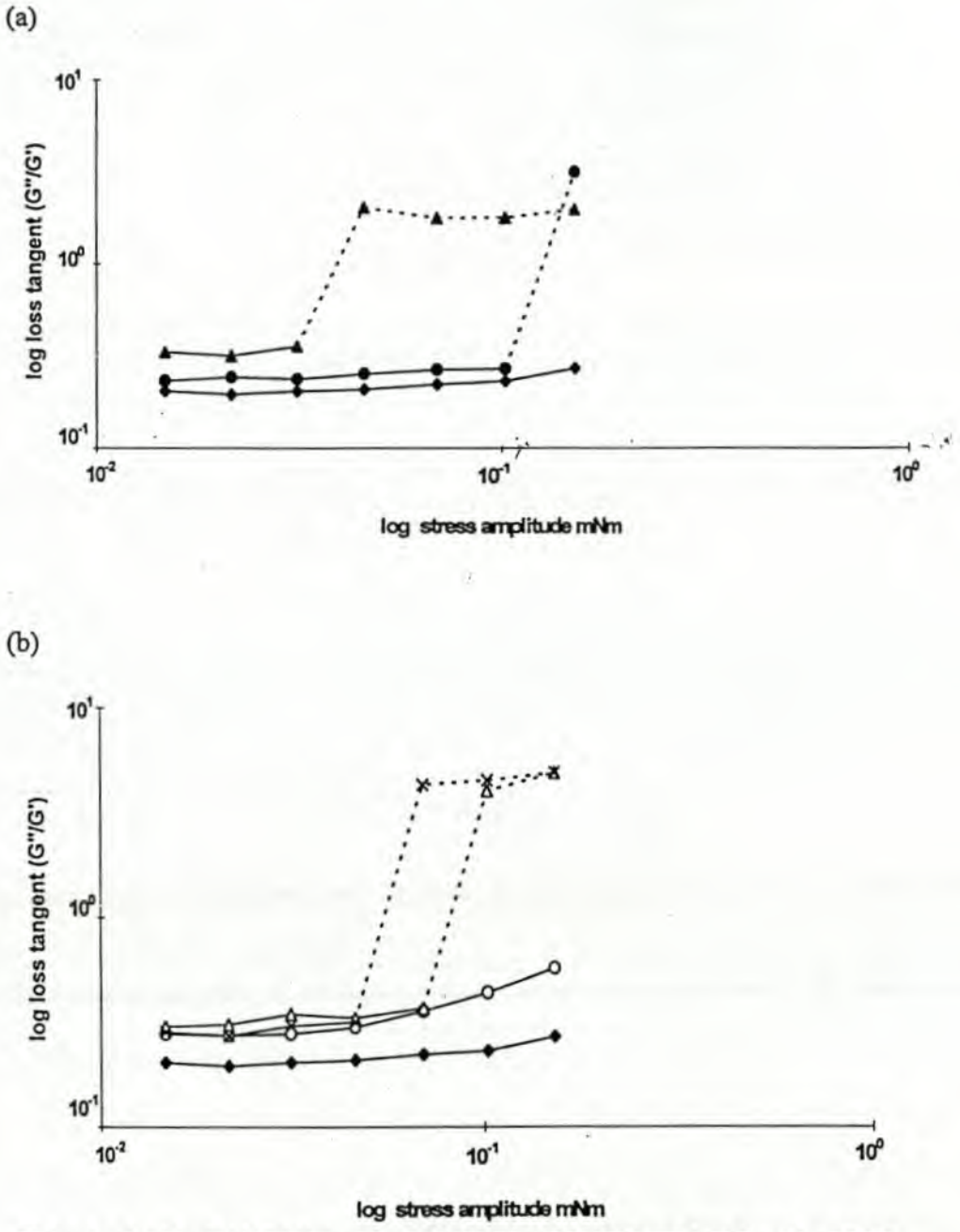

FIG. 4. LOSS TANGENT OF SAMPLES CONCENTRATED (a) POST-FERMENTATION, (b) PRIOR TO FERMENTATION AS A FUNCTION OF AMPLITUDE SWEEP

Standard deviations are less than symbol dimensions, (♦) control, (▲) RO-after fermentation labneh, (●) UF-after fermentation labneh. (△) RO-before fermentation labneh, (○) UF-before fermentation labneh, (×) Direct reconstitution labneh.

that incubation continued during membrane applications. In UF-after fermentation labneh, along with the increase in the protein level, the distribution of the protein particles may have been regulated by UF application. Mild pressure driven

effect of UF may have led to a more homogeneous distribution of protein network and, consequently, stress carrying strands over the continuous phase. It is fair to believe that UF application to warm yoghurt at low pressure (4 bar) may stimulate the increase in the number of smaller conglomerates and, therefore, number of stress carrying effective bonds. On this application, protein-protein bonds which are jammed in a large conglomerate, may be distributed homogeneously without disturbing the gel. Yet, RO application to warm yoghurt at high pressure (20 bar) caused an unrecoverable damage in the gel structure of RO-after fermentation labneh, giving in it an atypical appearance for labneh.

UF-before fermentation had significantly higher storage modulus (G′) than direct reconstitution and RO-before fermentation labnehs. This is probably due to the variation in the protein contents (9% vs 6.38–6.82%, respectively). As a result of more protein-protein interactions at higher protein levels, a much denser and stronger gel structure can be expected. This is seen in Fig. 5 showing the effect of protein level on the network moduli. Protein levels were adjusted from 1 to 9% by diluting the UF-milk having 10% protein content with the same permeate in order to keep the ionic strength relatively equal in each sample. An increase in both moduli with the increase in protein level is seen. A critical protein level had to be exceeded to form a gel (minimum 3% protein). In general, network moduli in a gel are more related to the particle concentration, however, the phase angle values are more dependent on the nature of bonds between these particles. In the present case, in spite of the UF-before fermentation labneh had much higher G′ and G″ values, insignificant differences were obtained between the same samples ($P > 0.05$).

The differences in the overall gel strength at low amplitude suggests that, although the type of protein-protein interactions (mainly whey protein/κ-casein interactions) in each case may be similar, there are differences in the degree of interaction. Subsequent breakdown at higher amplitudes suggests that the overall domains of the proteins may have been reduced and that there was a different "space occupancy" of the proteins in the gel material. The breakdown as a function of amplitude persisted even during storage of the materials for up to 14 days, suggesting that any changes produced during nontraditional methods of manufacture were essentially permanent and nonreversible.

CONCLUSION

Labneh is a typical weak viscoelastic gel whose properties are strongly affected by the methods of manufacture. Different gel behaviour at lower and higher amplitudes indicates that the type of the interactions that lead to the gel network are similar, but degrees of both covalent (SH/S-S exchange) and noncovalent

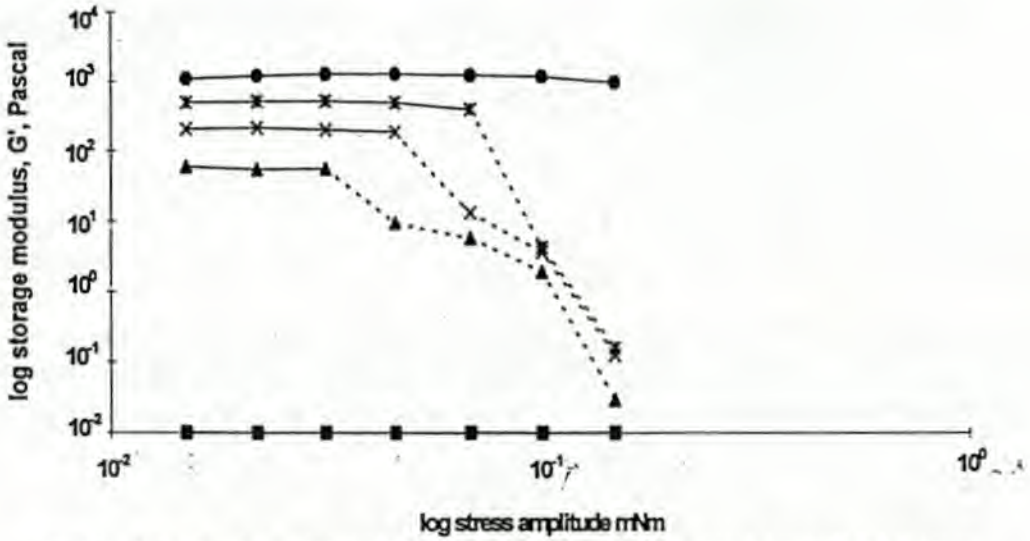

FIG. 5. EFFECT OF PROTEIN CONCENTRATION ON THE GEL PROPERTIES OF LABNEH MADE FROM UF MILK

No gel formed at the protein concentration less than 3%. Standard deviations are less than symbol dimensions. (■) 1%, (♦) 2%, (▲) 3%, (×) 5%, (*) 7%, (●) 9% protein.

(hydrophobic, electrostatic) interactions may be different as a result of the difference in protein level. The share of different gel-forming interaction forces in the formation of labneh gels is being currently investigated. Preliminary results indicate there are some differences between the samples regarding the level of protein interactions, especially the level of thiol-disulfide exchange reactions between whey proteins and κ-casein. Overall, the results suggest that UF applications can be used as an alternative method to the traditional labneh-making process (confirming the proposal of Tamime *et al.* 1989). The other production methods did not give gel properties that were close to those of typical labneh material.

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Effect of protein concentration on the properties and structure of concentrated yogurts

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Concentrated yogurts were produced by traditional (control), direct reconstitution, ultrafiltration and reverse osmosis techniques. The membrane techniques were applied either before or soon after incubation. The physical properties of the samples were monitored using a penetrometer (set yogurt) and viscometer (stirred yogurt), and the results indicated that different manufacturing techniques led to differences in the rheology of the concentrated yogurts. As expected, samples with high protein contents had greater gel strengths. Also, the concentration techniques caused large differences between the samples, even at the same protein level. The rheological properties correlated well with the microstructure as monitored by confocal laser scanning microscopy. In general, larger compartments in the network were associated with a weaker structure.

INTRODUCTION

Yogurt gels are particulate structures, mainly composed of caseins.[1] Their structure has been studied widely, as have the effects of processing conditions, such as heat treatment, type of starter culture or level of total solids, on the rheology of both set and stirred yogurts.[2–4] In addition, the formation of yogurt gels has been monitored using electron microscopy and dynamic oscillatory rheometry.[5,6] Depending on the processing conditions, continuously connected protein strands produce a heterogeneous three-dimensional gel network which holds free water. Any factors which affect the properties of the gel network by changing the nature and number of protein interactions will also affect the water holding capacity of the gel. The gel structure is known to involve both covalent (thiol/disulfide interchange) and non-covalent bonds.[7] Dickinson[8] claimed that the physical characteristics of particulate gels are determined by both strong permanent bonds (covalent bonds) formed during the aggregation, and subsequent rearrangements of protein particles. Furthermore, the final gel structure is also dependent upon the nature of weak reversible interactions between the particles prior to formation of the permanent bonds; therefore, the balance between strong and weak bonds controls the rheology.[8] Another factor affecting the physical characteristics of yogurt type gels is the distribution of protein–protein bonds over the gel network.[9] Several studies have investigated the relationship between protein concentration, distribution of protein–protein bonds and rheology of resulting gels.[10,11] In the case of homogeneous cross-linked particulate gels, all particles contribute to the network moduli equally.[11] However, in non-homogeneous gels like yogurt thick protein nodes including more than one protein junction point are evident, and their contribution to the elasticity of a gel decreases as the number of stress carrying strands decreases.

The use of membrane techniques in the manufacture of concentrated yogurt has gained popularity,[12] but knowledge of the gel characteristics of concentrated yogurts is limited.[13–15] Consequently, the aim of the present study was to prepare concentrated yogurts by different methods, and determine whether apparent contrasts in rheology would be confirmed by confocal laser scanning microscopy.

MATERIALS AND METHODS

Materials

Full cream milk powder (Adams Food Ingredients Ltd, Leek, Staffs) was used in the production of all the yogurts, as was a freeze dried yogurt culture (coded-CH1) from Chr Hansen's Laboratory (Reading, UK). The starter was a blend of *Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp *bulgaricus* in equal proportions, and resuscitation and routine subculturing were carried out according to the procedure described by Tamime.[16]

Methods

Yogurt was manufactured according to the method described by Ozer *et al*,[14] and the standard milk base (16 g l^{-1} total solids (TS)) was prepared by reconstituting the required amount of milk powder in tap water. Six different concentration techniques were applied

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in order to generate two groups of concentrated yogurts, namely set (concentration before fermentation) and stirred (concentration after fermentation):

Traditional concentrated yogurt (control) was produced by holding batches of yogurt (5 kg, 16 g l^{-1} TS) in bags of double layer cheese cloth at 4°C for 18–20 hours.

Concentrated yogurt (direct reconstitution) was manufactured by reconstituting the required amount of milk powder in water at 40°C to give 5 kg of endproduct with 23 g l^{-1} TS.

Concentrated yogurt (ultrafiltration (UF)-after fermentation) 16 g l^{-1} TS fermented milk (pH 4.3) was concentrated by UF at 42°C immediately after fermentation. The pH value of finished product was around 4.0.

Concentrated yogurt (UF-before fermentation) was made by concentrating freshly reconstituted milk (16 g l^{-1} TS) to approximately 23 g l^{-1} TS at 50°C prior to fermentation.

Concentrated yogurts (reverse osmosis (RO)-after and before fermentation) were prepared as the UF products except that RO systems replaced UF.

Both UF and RO were carried out using tubular systems supplied by Peterson Candy International (PCI Membranes, Whitchurch, Hants, UK). The UF membranes were ES 625 (polyether sulfone), surface area 0.8 m^2, MWt cut-off 25000 Da, operated at inlet and outlet pressures of 0.3 and 0.1 MPa respectively. The RO membranes were ZF 99 (polyether sulfone), surface area 1.2 m^2, operating at a pressure of 2 MPa.

All the samples (~ 150 g) were dispensed into (stirred yogurt) or incubated in (set yogurt) polystyrene cartons, and the pH values of the samples were measured using a pH meter (model Kent EIL 7045/46) fitted with a standard combination of glass electrode.

Assessment of the physical properties

The gel strength was measured with a standard penetrometer (Stanhope Seta Ltd, Camberley, Surrey, UK) using a probe of 67 g, a diameter of 2.5 cm and an apical angle of 90°; the penetration time was 5 s. In all cases, the temperature of the yogurt was equilibrated at 10°C, and duplicate pots from each batch were assessed.

After standardizing the temperature at 10°C, the apparent viscosity was measured with a Brookfield Viscometer (Model LVT with Helipath Attachment) (Brookfield Engineering Inc, Stoughton, USA) fitted with a T-bar spindle (D) rotating at 0.6 rotations per minute; the readings were converted to centipoises using the factor supplied by the manufacturer.

Statistical analysis

The results were analysed in Exel (Windows 95) to obtain mean values and standard errors, and the physical properties were compared with a standard *t* test.

Confocal laser scanning microscopy

Yogurt samples were prepared at least 24 h prior to examination, and a thin 'slice' taken with a scalpel was placed on a clean slide. After staining for 5–10 minutes with 0.3% fast green (BDH/Merck, Poole, UK), the slide was examined under a Zeiss LSM II Confocal laser scanning microscope (Zeiss, Welwyn Garden City, UK) using oil immersion optics (numerical aperture = 1.41). The protein was imaged by excitation of the fast green using a helium/neon laser emitting at a wavelength of 633 nm.

RESULTS AND DISCUSSION

The chemical compositions of test samples are given in Table 1. In general, while UF treatment to both fresh milk and fermented milk led to an increase in the protein and fat content, a large decrease in lactose was seen. However, in the RO treated samples and direct reconstitution concentrated yogurt, the concentrations of all the components rose proportionally.

The comparative gel strengths and viscosities are shown in Table 2, and it was notable that the gel strength of the traditional product, although stirred after leaving the cloth bag, was much higher than the rest. However, by contrast, the product made from milk concentrated by UF prior to fermentation gave

TABLE 1

Chemical compositions of the reconstituted milk and the concentrated yogurts. Results are the means and standard errors of duplicate samples taken over three separate runs and expressed as g kg^{-1} of sample

Samples	*Total solids*	*Protein*	*Lactose*[a]	*Fat*	*Ash*
Milk base	160.0 ± 1.8	43.6 ± 0.08	61.6 ± 0.9	45.0 ± 1.5	9.8 ± 0.3
Traditional yogurt	233.1 ± 2.0	92.0 ± 1.4	41.6 ± 0.5	91.8 ± 1.0	7.9 ± 0.5
UF-after fermentation yogurt	226.4 ± 3.3	88.0 ± 2.4	45.3 ± 3.4	84.5 ± 1.0	8.6 ± 0.2
RO-after fermentation yogurt	222.2 ± 1.5	63.8 ± 1.0	82.4 ± 2.2	66.0 ± 3.0	10.0 ± 0.5
UF-before fermentation yogurt	222.4 ± 2.1	90.0 ± 1.1	42.6 ± 0.9	82.0 ± 1.3	7.8 ± 0.1
RO-before fermentation yogurt	232.2 ± 10.3	68.2 ± 4.8	90.7 ± 0.2	62.5 ± 0.7	10.8 ± 0.3
Direct reconstitution yogurt	225.0 ± 1.9	63.8 ± 3.4	87.2 ± 3.1	61.0 ± 1.4	13.0 ± 0.2

[a]Lactose was determined by difference.
After Ozer *et al.*[13]

TABLE 2

Physical properties of the different yogurts measured as viscosity (centipoise/1000) and gel strength (penetration in mm) after overnight storage at 4°C

Product	*Viscosity*	*Gel strength*
Traditional	65.80 ± 1.2^{a}	78.30 ± 9.3^{a}
UF-after ferment.	27.50 ± 1.5^{b}	33.10 ± 1.3^{b}
UF-before ferment.	60.10 ± 1.0^{a}	31.70 ± 1.7^{b}
RO-after ferment.	5.60 ± 0.6^{c}	25.00 ± 1.0^{b}
RO-before ferment.	13.90 ± 1.0^{d}	30.30 ± 0.5^{b}
Direct Reconstit.	13.20 ± 0.9^{d}	28.30 ± 3.4^{b}

Each reading is the average of three separate trials, and means within a column sharing a common superscript do not differ significantly ($p > .05$).

a viscosity measurement close to the traditional, and the micrographs (Figs. 1a and 1d) showed that the two samples were similar, especially in terms of the size and structure of the voids. Exactly why the gel strength of the UF-before fermentation sample was significantly lower than the traditional product was not established, but it may have been because the cloth bag method allowed for the establishment of more protein–protein bonds. Thus, once the 5 l of yogurt were poured into the bag, it would have taken several hours for the temperature to reach 4°C and during this time the physical compression of the protein might have encouraged aggregation of the casein micelles and increase the extent of chemical bonding. In the membrane treated milk, by contrast, the extent of bonding would have been no different from that in any high solids yogurt, which would explain the similarity in gel strength between all the membrane concentrated yogurts.

In the absence of stabilizers, viscosity depends on total protein concentration and the size of the whey filled spaces, and there was an excellent visual correlation between viscosity and the dimensions of the void spaces (see Fig. 1). Thus the dense protein networks of the traditional and UF-before fermentation yogurts confirm the expectation of high viscosity, while the damage to the gel inflicted by membrane processing, especially by the high pressure associated with the RO treatments led to considerable breakdown of the gel structure (see viscosity measurements in Table 2) and the emergence of large whey filled spaces (Figs. 1c and 1e).

A contrast between the gels with different protein levels was apparent also for, in general, as the total solids increased, so the chains of casein particles became shorter, the dimensions of the voids diminished and the density of the matrix increased.[17] In the traditional UF-after and UF-before fermentation concentrated yogurts (Figs. 1a, 1b and 1d) much denser structures were observed compared to samples with lower protein contents (the RO-before fermentation, RO-after fermentation and direct reconstitution concentrated yogurts (Figs. 1c, 1e and 1f).

Overall, the study confirmed that damage done to the coagulum has a major impact on the viscosity of concentrated, stirred yogurts, and that the larger the undisturbed aggregations of casein and smaller the whey filled spaces, the higher the viscosity of the endproduct. Gel strength, by contrast, is probably more dependent on the extent of protein–protein bonding, and factors that may encourage these interactions are clearly important.

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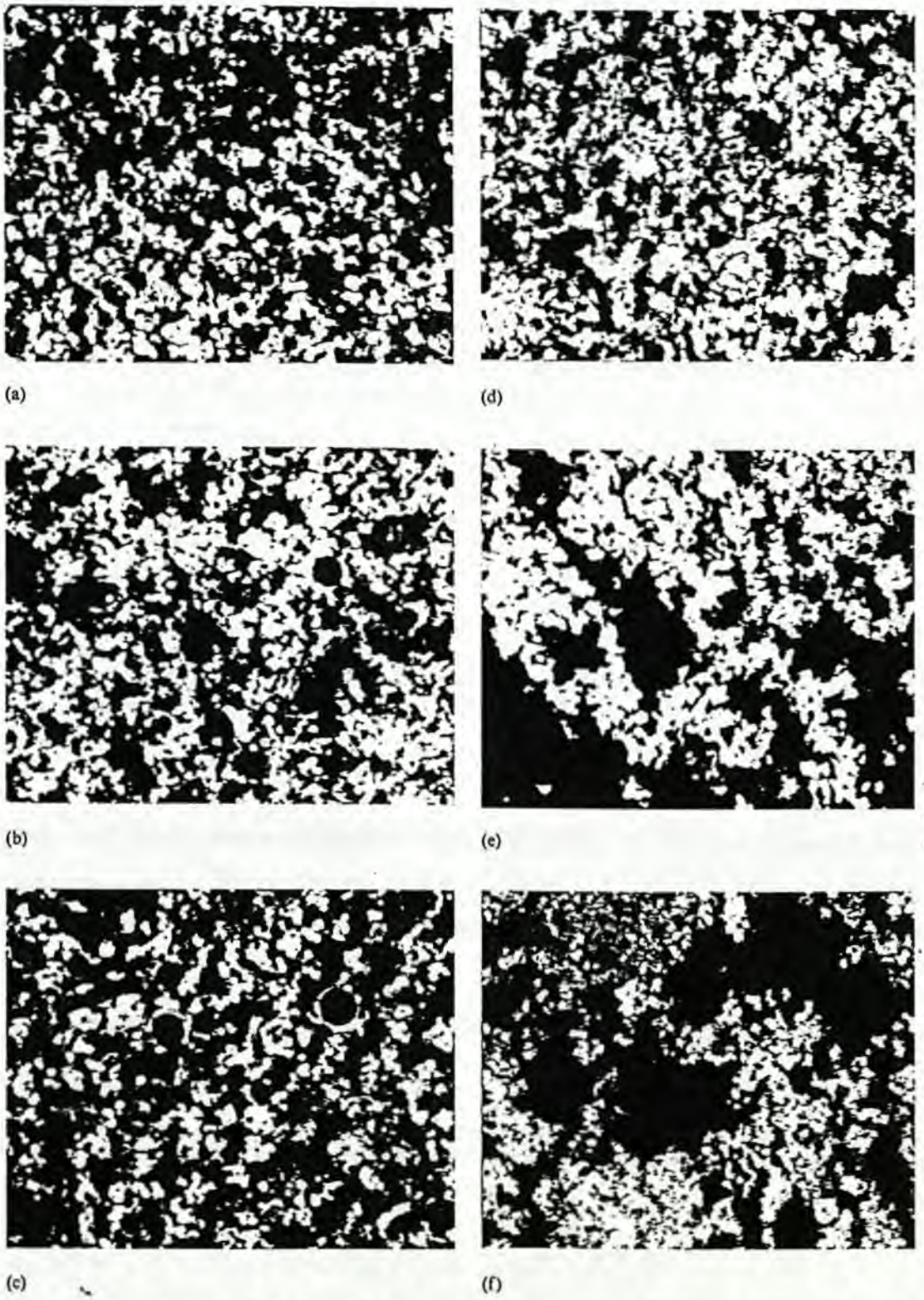

Fig. 1. Confocal laser scanning micrographs of protein distribution in stirred concentrated yogurts (23 g l^{-1} total solids). The protein was stained with fast green and imaged by excitation at 633 nm. Whey filled voids in the structure were seen by negative contrast. The circular voids are fat globules, and this conclusion was confirmed by staining with nile-blue with excitation at 488 nm. The samples were: (a) traditional product, (b) UF-after fermentation, (c) RO-after fermentation, (d) UF-before fermentation, (e) RO-before fermentation and (f) direct reconstitution.

Rheology and Microstructure of Labneh (Concentrated Yogurt)

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ABSTRACT

Labneh was produced by concentrating milk to approximately 23% total solids (wt/vol) by the traditional cloth bag method (control), ultrafiltration, reverse osmosis, or direct reconstitution. For ultrafiltration and reverse osmosis, membrane processing was carried out either before or immediately after fermentation. Dynamic rheological studies revealed that the physical behavior of labneh was heavily dependent on the protein concentration and the severity of mechanical agitation during membrane treatment. Scanning electron microscopy showed that the higher protein content samples had more compact structure and smaller voids than their lower protein content counterparts. Also, reverse osmosis and ultrafiltration of warm fermented milk had clearly detrimental effects on gel structure, producing thicker casein strands than in the traditional sample. No major differences were observed in the other test samples except gel densities, which varied with the different casein concentrations. In general, ultrafiltration of warm, fermented milk is a promising treatment for the manufacture of good quality labneh. (**Key words**: rheology, microstructure, concentrated yogurt, labneh)

Abbreviation key: **G*** = complex modulus, **RO** = reverse osmosis, **RO-AF** = RO after fermentation, **RO-BF** = RO before fermentation, **SCEM** = scanning electron microscopy, **tan δ** = loss tangent, **UF-AF** = ultrafiltration after fermentation, **UF-BF** = UF before fermentation.

INTRODUCTION

There have been numerous studies on the relationships between structure and texture of yogurt and yogurt-like products (5, 6, 8, 9, 20). The rheological properties of natural set and stirred yogurts have been widely investigated (14, 17, 18) as have the effects of processing variables such as heat treatment, concentration of TS, and type of starter bacteria on rheology and microstructure (2, 12, 15). However, until recently, such rheological and microstructural examination had not been carried out with labneh, a concentrated yogurt that is popular in the Middle East and Balkan regions (19). The recent increase in the popularity of labneh in Europe has led to more interest in the structure of labneh, especially in relation to milk species and concentration techniques (19, 21). In those studies, UF was proposed as a better alternative to the traditional labneh-making process, which is uneconomical and unhygienic (19). Recent studies in that laboratory (10, 11) have investigated the rheology of labneh produced by a range of techniques for increasing TS and have concluded that UF could be used to produce gel properties similar to the traditional product. The present study aims to extend that work by comparing the physical and microstructural properties of labneh produced by traditional methods, direct reconstitution, and membrane techniques [both UF and reverse osmosis (**RO**)] using dynamic rheological techniques and scanning electron microscopy (**SCEM**). In addition, a separate experiment was carried out in which labneh was prepared from UF retentates with varying protein contents. Rheological studies on these samples were used to assess the role of protein concentration regardless of method of concentration.

MATERIALS AND METHODS

Membrane Processing

Both UF and RO were carried out using tubular systems supplied by Paterson Candy International (PCI Membranes, Whitchurch, United Kingdom). The UF membranes were ES 625 (polyether sulfone) with a surface area of 0.8 m^2 and a molecular mass cutoff of 25,000 Da and were operated at inlet and

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outlet pressures of 0.3 and 0.1 MPa, respectively. The RO membranes were ZF 99 (polyether sulfone) with a surface area of 1.2 m^2; the operating pressure was 2 MPa.

Labneh Manufacture

Full fat, medium heat-treated milk powder supplied from Adams Food Ingredients Ltd. (Leek, United Kingdom) was used in the preparation of labnehs. The method of yogurt manufacture proposed by Tamime and Robinson (22) was applied. Milk powder was reconstituted at 40°C by a high speed mixer (Silverson Machines Ltd., Chesham, United Kingdom) to the desired TS concentration before further treatment. The milks were heated to 85°C for 20 min and then were cooled to 42°C in an ice-cold water bath. Samples were inoculated with starter culture (CH-1; Chr. Hansen's Laboratory, Reading, United Kingdom) at a rate of 2% (wt/wt). The starter culture was a blend of *Streptococcus thermophilus* and *Lactobacillus delbruckii* sp. *bulgaricus* in equal proportions. The desired TS content of the labneh samples at the end of the manufacture was approximately 23% (wt/vol), which is typical of traditional labneh from the Middle East (22). Incubation was halted when the pH dropped to 4.3 for samples that were concentrated postincubation and to 4.0 for previously concentrated samples; the final pH was 4.0 for all samples. Samples were stored overnight in 200-g plastic cups at 4°C and were allowed to equilibrate to 25°C in an incubation room before rheological measurements were performed the following day. Six different manufacturing methods were employed, and 8 replicates of each batch were prepared for analysis.

1. Traditional labneh. To produce traditional stirred-type labneh (control), yogurt with 16% TS (wt/vol) (pH 4.3) was drained after overnight refrigeration following manufacture in double layer cheesecloth bags until the desired TS concentration (~23%, wt/vol) was reached. Drainage was achieved at 4°C, and the volume of whey separated was measured periodically. Total drainage time was 18 to 20 h.
2. Stirred-type labneh produced by UF after fermentation (**UF-AF** labneh). Ultrafiltration was applied immediately after incubation of fermented milk (16% TS, wt/vol) was complete (pH 4.3). The temperature was maintained at 42°C by circulating cold water around the feed tank when necessary. After the desired concentration of TS was reached (~23% wt/vol), the sample was filled into yogurt pots and stored in the refrigerator until analysis.
3. Stirred-type labneh produced by RO after fermentation (**RO-AF** labneh). The procedure just described for UF-AF was followed except that RO was applied.
4. Set-type labneh produced by UF before fermentation (**UF-BF** labneh). Milk with 16% TS (wt/vol) was concentrated by UF to 23% TS (wt/vol) at 50°C. The standard yogurt manufacturing procedure was then followed. Incubation was stopped at pH 4.0.
5. Set-type labneh produced by RO before fermentation (**RO-BF** labneh). The same procedure described for UF-BF labneh was followed except that RO membranes were applied.
6. Direct reconstitution, set-type labneh. The procedure for the control was followed except that milk powder was dissolved to a concentration of 23% TS (wt/vol). Incubation was halted at pH 4.0.

The fermentation process took approximately 3.5 h for the control, UF-AF, and RO-BF labnehs and took 5 to 6 h for the direct reconstitution, UF-BF, and RO-BF labnehs.

After overnight refrigeration at 4°C, the pH values of the samples were around 4.0.

Gels with Varying Protein Concentrations

Milks were prepared with protein concentrations ranging from 1 to 9% by UF with appropriate dilution of retentates to the appropriate protein concentration with the same UF permeate to maintain the same ionic environment. Standard yogurt manufacture was carried out as just described.

Chemical Analysis

Protein, TS, fat, and ash were determined by the methods of the British Standards Institution (10).

Rheological Measurements

Rheological properties of labnehs after overnight storage at 4°C were monitored using an RTI controlled-stress dynamic rheometer (Rheo-Tech Int., Ltd.; Camtel Ltd., Royston, United Kingdom). The rheometer was set up with a parallel plate geometry (10-mm radius and 1-mm gap setting), and the temperature of the samples was maintained at 25°C by a circulating water system. Labneh samples were

evaluated rheologically by conducting stress and frequency sweep tests. The frequency and amplitude ranges were 10^{-3} to 10^{1} Hz at 0.07 mNm torque and 1.5×10^{-2} to 1.5×10^{-1} mNm at 0.25 Hz, respectively. Labnehs were kept in a room maintained at 25°C to equilibrate before being loaded into the rheometer. Samples were allowed to relax (5 min) prior to assessment of their amplitude and frequency behavior.

SCEM Studies

Preparation of thick sections for SCEM. Labneh samples were prepared for SCEM studies according to the method proposed by Brooker and Wells (4). Specimens of labneh were fixed and solidified by addition of 25% SCEM grade glutaraldehyde at a ratio of glutaraldehyde to labneh of 1:7 (wt/vol). This mixture was poured onto a petri dish as a thin layer and left for 30 min at 4°C to solidify before 1-mm cubes were cut with a razor blade. Because the RO-AF labneh remained too soft to handle after this treatment, the cubes were coated with 3% aqueous agar to protect them and were stored overnight in 3% glutaraldehyde in 175 m*M* sodium cacodylate-HCl buffer (pH 7.2).

After rinsing in water, the specimens were dehydrated with three changes of acidified dimethoxy propane over 3 h and embedded in araldite resin. Two-micrometer sections of labnehs were cut with a glass knife on an ultramicrotome (Reichert Ultracut E; Leica UK, Milton Keynes, United Kingdom), placed on a drop of 10% acetone on a circular 10-mm diameter cover slip, and dried by gentle heating.

Etching of sections. A stock solution of saturated NaOH in absolute ethanol was prepared by the method of Lane and Europa (7) and was allowed to stand for about 1 wk until the solution became dark brown. The coverslip was immersed in stock solution that had been diluted 1:1 (vol/vol) with ethanol immediately before the sample was placed on a hot plate (40°C) for 10 min. The progress of etching was followed using a binocular microscope. Complete removal of the resin was considered to have occurred when the boundary of the section was no longer visible.

Preparation of etched sections for SCEM. When etching was judged to have reached a desirable stage, the coverslips were transferred rapidly to ethanol (to prevent ethanol evaporation, which would lead to NaOH crystallization on the surface) and washed several times with absolute ethanol. The coverslips were then transferred to 100% acetone for 10 min before critical point drying (Polaron E3000; Polaron Equipment Ltd., Watford, United Kingdom) with liquid CO_2 and were mounted on aluminum stubs using silver conducting paint and then coated with gold in a vacuum sputter coater (Edwards High Vacuum S150; Crawley, Sussex, United Kingdom). The sections were placed 20 mm from the gold electrode and were sputtered for 1 to 3 min (depending on thickness) under a vacuum of 20 Pa using 2.5-kV high tension and a discharge current of 20 mA. Sections were examined in an SCEM (Hitachi L750; Nissei Sangyo Co. Ltd., Tokyo, Japan) operating at accelerating voltages from 5 to 30 kV. The results were recorded on Kodak plus X 120 film (Kodak Ltd., Hemelhempstead, United Kingdom).

RESULTS AND DISCUSSION

Chemical Composition

The chemical composition of the labnehs are summarized in Table 1. The traditional (control) and UF-

TABLE 1. Chemical composition of labnehs.[1]

Labneh[2]	TS		Protein		Lactose[3]		Fat		Ash	
	(mg/kg)									
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Traditional (control)	23.31	0.20	9.20	0.14	4.16	0.05	9.18	0.10	0.79	0.05
UF-AF	22.64	0.33	8.80	0.24	4.53	0.34	8.45	0.10	0.86	0.02
RO-AF	22.22	0.15	6.38	0.10	8.24	0.22	6.60	0.30	1.00	0.05
UF-BF	22.24	0.21	9.00	0.11	4.26	0.09	8.20	0.13	0.78	0.01
RO-BF	23.22	1.03	6.82	0.48	9.07	0.02	6.25	0.07	1.08	0.03
Direct reconstitution	22.50	0.19	6.38	0.34	8.72	0.31	6.10	0.14	1.30	0.02

[1]n = 3.

[2]Labneh manufactured with UF-AF = (UF after fermentation), UF-BF = (UF before fermentation), RO-AF = [reverse osmosis (RO) after fermentation], or RO-BF = (RO before fermentation).

[3]Determined by difference.

treated labnehs (UF-AF and UF-BF labnehs) had higher protein and fat contents than did the remaining test samples, which was to be expected as the double-layer cloth bags and UF membranes allowed the separation of lower molecular mass compounds, such as lactose and minerals, into the permeate or filtrate while retaining protein and fat. Conversely, with RO and direct reconstitution, all constituents were concentrated in proportion to the concentration factor. The latter samples had much higher lactose contents than the traditional and UF-treated samples. There were no major differences in chemical composition between labnehs that were concentrated before and after fermentation.

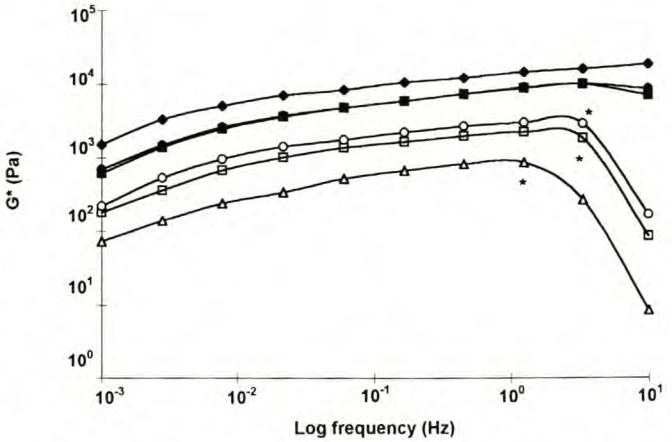

Figure 1. Frequency sweep pattern of labnehs tested. Results are the means of eight separate runs repeated five times (n = 8). Standard errors are less than ±8% of the mean values and smaller than symbol dimensions. Asterisks indicate the complex modulus (G*) after gel structure degradation (outside the linear viscoelastic region). Traditional labneh (♦; control); labneh manufactured with reverse osmosis (RO) after fermentation (△), UF after fermentation (■), UF before fermentation (●), direct reconstitution (○), and RO before fermentation (□).

Dynamic Rheological Properties

The physical properties of the labnehs were examined by conducting frequency sweep tests (Figure 1). The different manufacturing techniques led to differences in the physical properties of the resulting products. Within the linear viscoelastic region, the traditional sample had the greatest complex modulus (**G***), followed by UF-AF and UF-BF samples, direct reconstitution, RO-BF, and RO-AF labnehs, respectively. Structural degradation occurred at some point in the frequency range in all except the traditional labneh. Both the rheological differences between the samples within the linear viscoelastic region and structural degradation indicate that the same interaction forces take place in the formation of labneh gels but to different degrees. Similarly, within the linear viscoelastic region, the loss tangent (**tan** δ = loss modulus/storage modulus) values of the samples were not significantly different, indicating that the nature and type of the interaction forces were similar (data not shown). The storage modulus and loss modulus are similarly related to the spatial distribution and the number of protein-protein bonds, which, therefore, suggests that tan δ is related to the nature of the protein bonds (13). A slight frequency dependency within the linear viscoelastic region was apparent, which seemed to be independent of the method by which the labnehs were manufactured. The increase of G* with frequency suggests a relaxation of bonds over the time scale of the measurements (13) with greater numbers of individual bonds relaxing over time. This type of rheological behavior is typical of particle gels such as yogurt (1).

A yogurt gel network is primarily built of casein and denatured whey protein complexes, and, to study the role of protein concentration (independent of method of concentration) in gel formation, UF milks were adjusted to various protein concentrations using the same UF permeate to keep the same ionic environment of each sample. The effect of protein concentration on the dynamic modulus G* and tan δ is illustrated in Figure 2. A strong dependence of G* and tan δ on the protein concentration was evident. The minimum protein concentration at which a gel could be formed was 3%, which is presumably due to the lower number of protein contacts in the larger solvent concentration. Structural degradation occurred at protein concentrations up to 7% but not at 9%. With increased protein, G* increased. These findings are consistent with previous studies (3, 16, 23), which relate gel strength to the casein concentration. Bremer et al. (3) proposed that the total length of the stress carrying strands per unit volume is a decisive factor for the gel strength of yogurt and that the nature and position of the strands in the network also determine the rheological properties of the gel. Ross-Murphy (16) stated that, ideally, there should be a direct relationship between the number of molecules participating in the junction zones of a protein network and the gel strength. Also, Walstra et al. (23) reported that the number of contact points between the casein clusters is independent of their size but dependent on casein concentration.

As can be seen from Figure 2b, within the linear regions, tan δ values were independent of casein (pro-

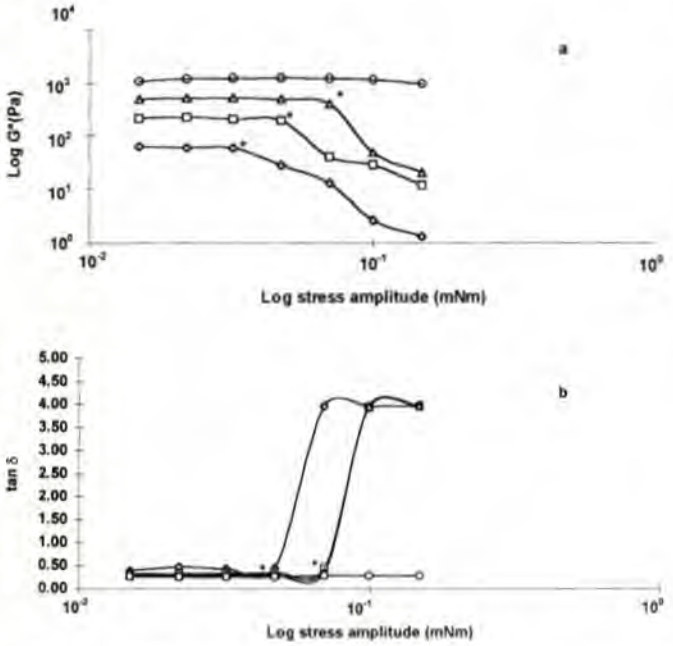

Figure 2. Effect of protein concentration [3% (◊), 5% (□), 7% (△), or 9% (○)] on the complex moduli (G*) (a) and the loss tangent (tan δ) (b) of the samples examined. Results are the means of eight separate runs repeated five times (n = 8). Standard errors are less than ±8% of the mean values and are smaller than symbol dimensions. Asterisks indicate the complex modulus (G*) after the gel structure has degraded (outside the linear viscoelastic region).

tein) concentration, which implies that the nature of the protein-protein bonds was the same. Because the dependence of G* on the protein concentration was nonlinear, it can be concluded that the number of stress-carrying strands was not proportional to volume fractions of casein particles, and so the network formed was very heterogeneous.

Microstructure

The SCEM of traditional and stirred-type labnehs are presented at low and higher magnifications in Figures 3 and 4, respectively, and the set-type labnehs are shown in Figure 5. The microstructure of the samples was determined by the mechanical treatments and the order of application of concentration technique (before or after fermentation). At low magnification (2500×), the traditional labneh (Figure 3a) appeared to have a more compact structure than did the other two stirred-type labnehs (UF-AF and RO-AF labnehs; Figure 3, b and c, respectively). Surprisingly, SCEM failed to reveal any notable difference between the two membrane-treated, stirred-type labnehs (Figure 3, b and c) despite the considerable

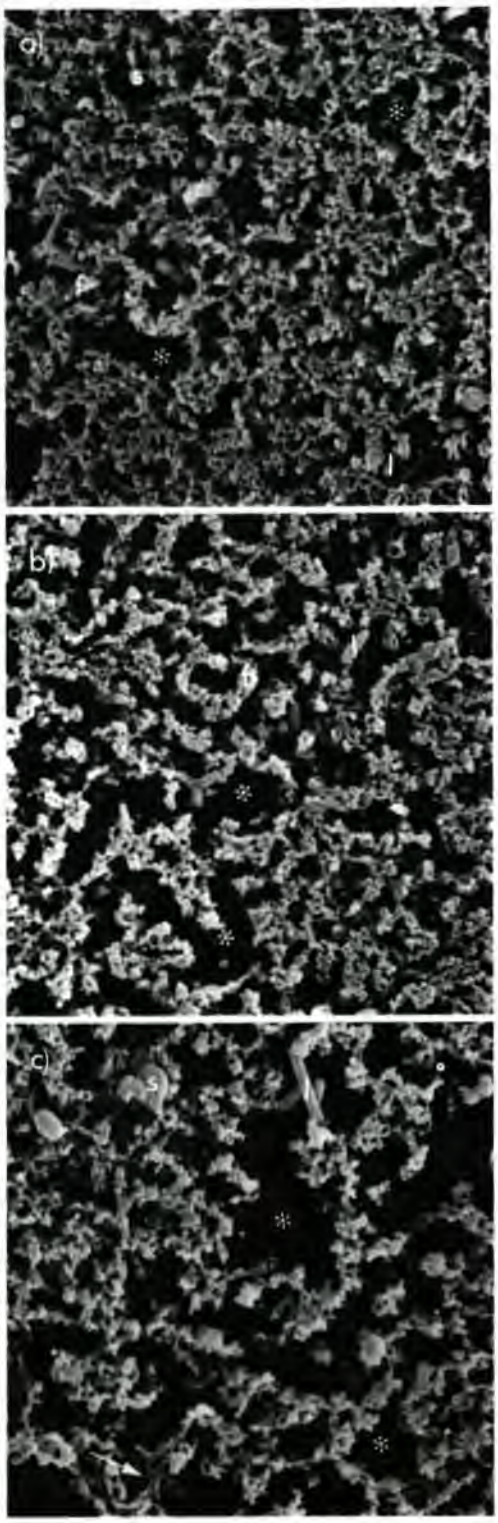

Figure 3. Scanning electron micrographs of stirred-type labnehs: traditional (control) (a), manufactured by UF after fermentation (b), and manufactured by reverse osmosis after fermentation (c). Continuous casein micelles (black arrows) and thread-like structures (white arrows) can be observed between casein strands. Magnification: 2500×. Legend: lactobacilli = l, streptococci = s, fat globules = f, and voids = *.

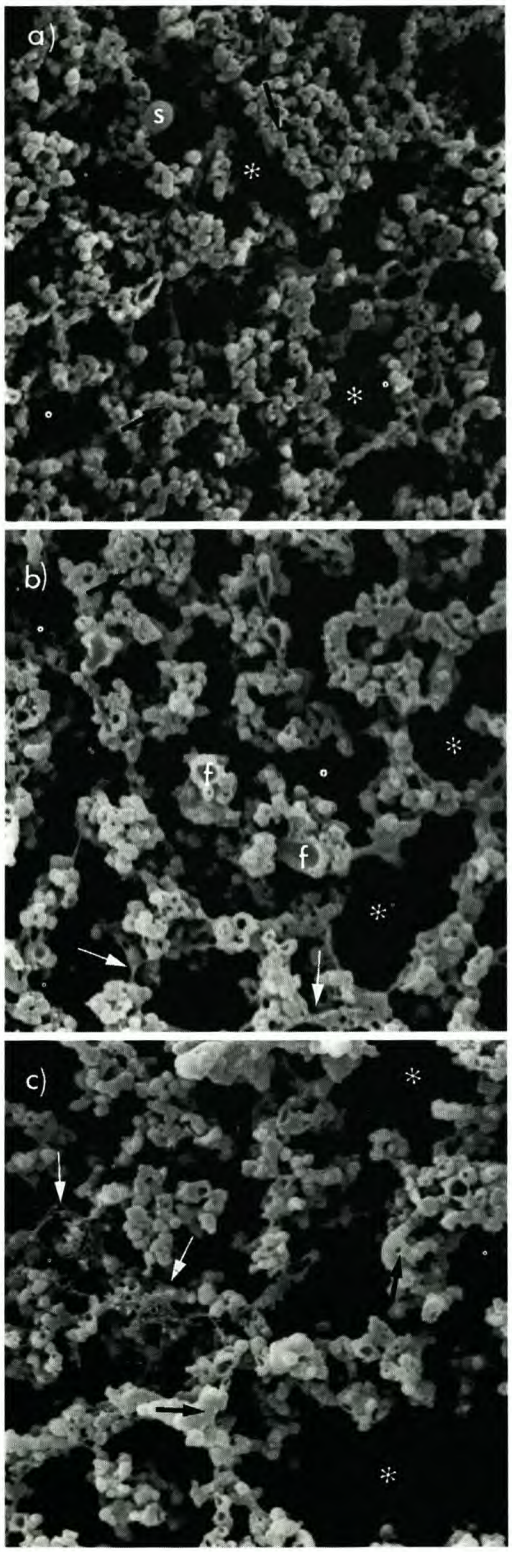

Figure 4. Scanning electron micrographs of stirred-type labnehs: traditional (control) (a), manufactured by UF after fermentation (b), and manufactured by reverse osmosis after fermentation (c). Continuous casein micelles (black arrows) and deformed (ripped-off) casein strands (white arrows) are evident. Magnification: 12,600×. Legend: streptococci = s, fat globules = f, and voids = *.

differences in the rheological properties and protein concentrations of these samples.

The RO-BF labneh had a structure similar to the direct reconstitution labneh (Figure 5) and is not shown. Damage was not detected in the structures of

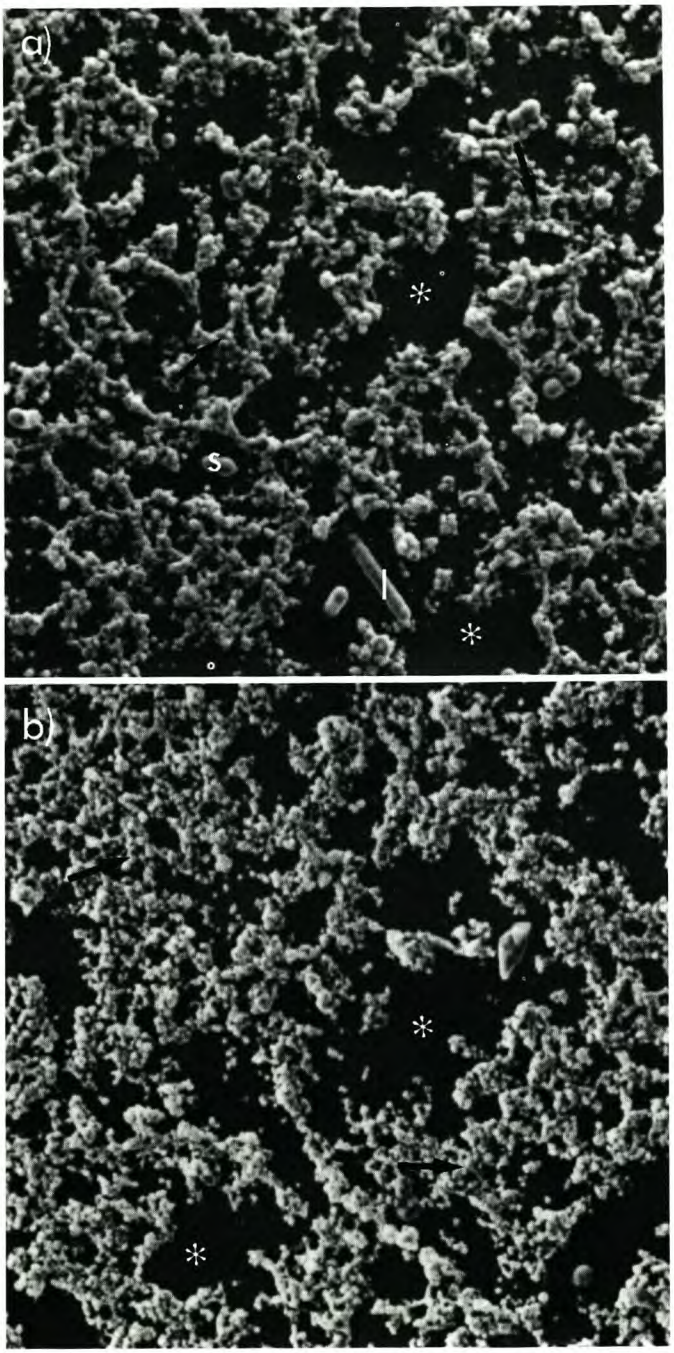

Figure 5. Scanning electron micrographs of set-type labnehs: manufactured with UF before fermentation (a) or by direct reconstitution (b). Continuous nondeformed structure is evident (black arrows). Magnification: 2500×. Legend: lactobacilli = l, streptococci = s, and voids = *.

UF-BF and direct reconstitution labnehs; the voids and protein structure were relatively evenly distributed.

The labnehs generally displayed continuity of structure except for RO-AF and, to a lesser extent, UF-AF labneh. The discontinuity in the membrane-treated, stirred-type labnehs is probably linked to the detrimental effect of RO and UF on the delicate gel structure. The casein clusters were thicker in the membrane-treated samples, perhaps as a result of pressure forcing the casein aggregates to come together during the early stages of the membrane processes. This compression is particularly notable at higher magnification in Figure 4, a and b, which shows that the thicker casein strands in UF-AF labneh occupied less space in the casein network than did the traditional product, although the protein concentrations were the same. However, the same membrane treatments to milks (i.e., before fermentation) did not produce such structures; instead, fine, continuous microstructures were evident (Figure 5a).

Small, thread-like structures were visible between the strands in both the RO-AF and UF-AF labnehs, which may be the result of stretching and shearing of casein aggregates during the later stages of UF and RO when viscosity is increased. More separate particles were present in the network in the RO-AF labneh than were in the UF-AF counterpart. The lower protein content of the RO-AF labneh would be expected to have resulted in considerably larger compartments compared with the higher protein content samples (traditional or UF-AF). However, fairly close network densities were evident in both membrane-treated, stirred labnehs (UF-AF and RO-AF Figure 3, b and c, respectively). One explanation is that the high pressure RO application to fermented milk might have caused a ripping of the casein aggregates, but the lower pressure during the UF process might have stretched the casein strands and broken a lower number of bonds between the casein aggregates. This difference may be connected to the wide polysaccharide-like structures in the UF-AF labneh compared with the tiny thread-like structures in the RO-AF seen at higher magnification (Figure 4, b and c, respectively). Because nonpolysaccharide-producing yogurt culture was used in the manufacture of the labnehs, these structures are unlikely to be polysaccharide materials.

It is possible that these unusual structures may be the result of artifacts or that the apparently separate particles may in some cases actually be the front view of a casein chain that has a continuous structure. However, these structures were not seen in the traditional (control) labneh (Figure 4a), which was not agitated, and there was reasonable agreement between the rheology and microstructure of samples tested. It is, therefore, concluded that the separate particles seen in the membrane-treated samples could be broken protein aggregates (UF-AF labneh) and broken or ripped-off casein strands (RO-AF).

CONCLUSIONS

The large differences in the rheological properties of the labnehs seemed to be dependent on the level of protein and TS elevation technique. Electron microscopy revealed that the application of the concentration techniques to the fermented milk had detrimental effects on the structure. The amount of damage to the casein strands seemed to be related to the shearing effect of the UF and RO membranes. However, in terms of the physical properties of the labnehs, the UF-AF and UF-BF labnehs had similar characteristics. Through changes in the processing variables (e.g., pH at the beginning of concentration, transmembrane pressure, and operating temperature), a material might be produced that has a less damaged structure and a better texture. In summary, UF treatment to the warm fermented milk seems to be a promising technique in the manufacture of good quality concentrated yogurt.

ACKNOWLEDGMENT

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Gelation Properties of Milk Concentrated by Different Techniques

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ABSTRACT

The rheological properties of three different concentrated yoghurts (~23% total solids, pH 4.0) and a standard yoghurt (~16% total solids, pH 4.3) were determined during incubation and storage. The high solids yoghurts were manufactured from milks concentrated by ultrafiltration (UF) to 9% protein, by reverse osmosis (RO) to ~6.8% protein and by direct reconstitution of full-fat milk powder to ~6.4% protein; the standard product contained ~4.3% protein. Development of the gel structures during incubation was monitored discontinuously using a stress-controlled oscillatory dynamic rheometer. The complex modulus (G^*) and loss tangent ($\tan \delta$) were measured as functions of amplitude (torque range 10^{-3}–10^{-2} mNm at 0.25 Hz). In the RO- and direct reconstitution yoghurts, a typical pattern of gelation was observed, with the initial viscous nature of the milk being modified by rapid development of a gel structure and, finally, a 'stationary phase' with respect to rheological changes. However, the complex modulus (G^*) of the higher protein system (UF-milk) continued to increase with time, i.e., further development of the gel structure occurred during storage. The relative rates of gel development were dependent on protein content.

Keywords: concentrated milk; gelation; rheology; dynamic moduli

INTRODUCTION

Yoghurt is defined as a cultured milk product produced with thermophilic lactic acid bacteria, usually *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Davies *et al.*, 1978; Kalab *et al.*, 1983; Tamime and Robinson, 1985; Mottar *et al.*, 1989). These starter bacteria, by converting lactose to lactic acid, are responsible for the reduction in zeta potential that leads to the destabilisation of the casein micelles and, incidently, the partial dissociation of calcium phosphate associated with the micelles; solubilisation is almost complete at around pH 5.2–5.0 (Heertje *et al.*, 1985; Visser *et al.*, 1979). As a result of a further decrease in pH, the casein micelles lose their structural integrity and become unstable (van Vliet *et al.*, 1991). After initial contact between the casein micelles, there is a loss of structural entropy which is accompanied by further aggregation and, under quiescent conditions, a three-dimensional heterogeneous casein network forms (Benezech and Maingonnat, 1994) held together by covalent and other protein–protein interactions (Dickinson, 1990; Rohm and Kovac, 1994). The extent of these interactions is determined by both the concentration and physico-chemical state of the proteins, as well as factors such as pH.

The heat treatment applied to the yoghurt milk further affects this basic process of gel formation; the usual treatment of 95°C for 7–10 min results in the denaturation and, subsequently, aggregation of the whey proteins (Euber and Brunner, 1982; Doi *et al.*, 1983; Hill, 1989). During this process, a specific interaction between κ-casein and β-lactoglobulin occurs via reduced thiol/disulphide interchange reactions (Hill, 1989), and these heat-induced protein interactions are essential for the formation of a yoghurt with desirable textural properties (Dannenberg and Kessler, 1988).

In commercial practice, many factors influence the physical characteristics of a yoghurt gel (Roefs, 1986; Roefs and van Vliet, 1990; Dickinson and McClement, 1996) and, while the importance of post-incubation conditions, such as in-plant shear and storage temperature, cannot be underestimated, it is believed that the rheological characteristics of yoghurt are determined mainly by the gelation process. This view is supported by a number of studies that have been carried out on gel formation during the incubation of standard yoghurt [16% total solids (TS), w/v] (Schulze *et al.*, 1991; Biliaderis *et al.*, 1992; Parnell-Cluiness *et al.*, 1988), but it is not clear whether the impact of the gelation process is so critical in milks of high total solids milks (22–23% TS, w/v) that might be used to manufacture concentrated yoghurt (Ozer *et al.*, 1997, 1998). Consequently, the aim of this study was to: (a) reconstitute bovine, full-fat milk powder to 16% TS, and then concentrate batches of milk by various techniques; and (b) monitor the physical properties of the milks throughout their fermentation to yoghurt, and during subsequent storage of the products at 4°C.

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MATERIALS AND METHODS

Materials

Throughout the study, a medium-heat, full-fat milk powder (whey protein denaturation, ~10%) supplied by Adams Food Ingredients Ltd. (Leek, Staffs., UK) was used as the basic raw material. The starter culture (Code CH1) was obtained in freeze-dried form from Chr. Hansen's Laboratory (Reading, England), and the suppliers stated that it was a blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in equal proportions.

Methods

Bottles (150 mL) of sterile milk (antibiotic-free, skim-milk powder reconstituted to 12% TS and autoclaved at 121°C for 2 min) were used to grow the starter culture on a routine basis, and the initial resuscitation was made by adding approximately 1 g to 100 mL of milk. After incubation at 42°C to obtain a weak gel, a subculture was made by transferring 2 mL of the original culture into 100 mL of sterile milk and incubating for 3.5 h. This routine was repeated weekly, and the fresh cultures were stored at 4°C prior to use.

Batches of milks (5 L, 22–23% TS, w/v) were prepared by three different techniques. One batch of milk was prepared by direct reconstitution of the milk powder to 23% TS (w/v) (direct reconstitution yoghurt), while for the second and third batches, 16% TS (w/v) reconstituted milk was further concentrated to approximately 23% (w/v) by ultrafiltration (UF-yoghurt) or reverse osmosis (RO-yoghurt). The membrane techniques were used at 50°C; the specifications of the UF membrane were: surface area, 0.8 m^2; type, ES 625; membrane material, polyether sulphone; nominal molecular weight cut-off, 25000 Da, and of the RO membrane: surface area, 1.2 m^2; type, ZF 99; membrane material, polyether sulphone. The UF cartridge consisted of a bundle of tubular membranes. Both membranes were supplied by Paterson Candy International (PCI, Whitchurch, Hampshire, England). The inlet and outlet pressures of the UF system were 0.3 and 0.1 MPa, respectively, and the working pressure of the RO system was 2 MPa. A final batch (unconcentrated control), prepared by reconstituting milk powder to 16% TS (w/v) was regarded as a 'control' because its rheological properties could be predicted from previous studies.

A Silverson High-speed mixer (Silverson Machines Ltd., Chesham, England) was used to incorporate the milk powder (16 or 23% TS) at 40°C and, after the necessary concentration, the milks were heated at 85°C for 20 min in stainless steel buckets placed in a water bath. Uniformity of heating was achieved by means of a low-speed (40 rpm) stirrer, and stirring was continued during cooling of the milk to 42°C in an ice-cold water bath. All batches were subjected to the same treatment and, although the thermal conductivities of the milks may have varied due to the different levels of salts and lactose, these contrasts did not appear to influence the rates of heat transfer; all samples reached 85°C within 15 ± 2 min.

The cooled milks were inoculated with the yoghurt starter culture (CH-1) at a level of 2% (w/w), and dispensed into yoghurt pots of 150 mL capacity. The pots were then incubated at 42°C until the desired pH value had been reached (pH 4.3 for the control and 4.0 for the concentrated samples). These values were selected because it is common practice to produce concentrated yoghurt at about pH 4.0, and normal yoghurt at pH 4.3 (Tamime and Robinson, 1985). The pH of the samples was recorded during incubation using a pH-meter (model Kent EIL 7045/46) fitted with a standard combination glass electrode, and ionic calcium was determined using an ion selective electrode ISE Ca^{2+}/pH Analyser Model 634 (Ciba Corning Company Diagnostic Ltd., Chelmsford, England); the rheological measurements were carried out as detailed below. After incubation to the desired pH, the samples were transferred to a cold room at 4°C.

Rheological measurements

The gelation profiles of the milks were monitored discontinuously using a RheoTech International (Camtel Ltd., Royston, Herts., UK) controlled-stress rheometer and, on any one occasion, only one type of yoghurt was examined. Samples of yoghurt were removed from the pots at half-hour intervals, from the first hour of incubation onwards, by taking a thin slice (1.0–1.2 mm) from beneath the surface of the gel, and loading it into the rheometer. The rheometer was set up with parallel plate geometry (10 mm radius chamber and 1 mm gap), and the temperature of the samples was maintained at 25°C using a circulating water system; this temperature was chosen to reduce the risk of a further decrease in pH during measurement. Each sample was loaded into the rheometer and allowed to relax and equilibrate to measuring temperature (5 min) prior to assessment of its rheological properties. The complex modulus (G) and loss tangent ($G^*/G^* = \tan\delta$) were determined as amplitude sweeps within a torque range 10^{-3} to and 10^{-2} mNm at 0.25 Hz during the gelation stage, and within a torque range 1.5×10^{-2} to 1.5×10^{-1} mNm at the same frequency for yoghurts during storage at 4°C.

A number of preliminary observations suggested that gelation occurred between 90 and 120 min. However, with the rheological technique employed in the present study, it was difficult to detect the exact onset of gelation since the measurements were recorded discontinuously and, on each occasion, a new cup was used; the pH and ionic calcium values were determined on the same sample. Consequently, the gelation point was taken as the point in time when the storage modulus (G') first exceeded the noise level of the measurement (~10 Pa). Each type of yoghurt was made on eight separate occasions for assessment of the rheological properties.

Statistical analysis

The results were analysed in Exel (Windows 95) to obtain mean values and standard errors, and gelation rates were compared using a standard t-test.

Rate of gel development

The rate of development was estimated by applying the first-order reaction kinetic model described by Biliaderis

et al. (1992):

$$\text{Gel development rate } (k) = 2.303/t \log \frac{(G^*_\infty - G^*_{t1})}{(G^*_\infty - G^*_{t2})}$$

where G^*_∞ = complex modulus at the completion of the gelation process, G^*_{t1} = complex modulus at the onset of gelation, G^*_{t2} = complex modulus at time t (minutes).

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the concentrated and control yoghurts are illustrated in Table 1. In general, the protein and fat contents increased in UF-yoghurt in line with the concentration factor, but the same fractions increased in the RO- and direct reconstitution yoghurts to a lesser extent. The lactose concentration was reduced to 4.26% (w/v) in UF-yoghurt from 6.16% (w/v), whereas in the RO-yoghurt and direct reconstitution milks, lactose increased with the concentration factor.

The pH value of the unconcentrated (control) milk (16% TS, w/v) reached pH 4.3 within 240 min but, in the higher total solids samples, the decline in pH was slightly slower due to the higher buffering capacity of the concentrated milks (data not shown).

All the samples showed a linear viscoelastic region (see Figs 1 and 2) with no sign of structural breakdown over the measured range (10^{-2}–10^{-3} mNm); no slippage was observed in the measuring system. Some structural damage could have occurred during loading of

Table 1. Chemical Composition of Yoghurt Samples. Results are the Average of Three Separate Runs ($n = 3$)

Samples	Total solids %	Protein %	Lactose[a] %	Fat %	Ash %
Unconcentrated yoghurt	16.00 ± 0.03	4.36 ± 0.04	6.16 ± 0.07	4.50 ± 0.6	0.98 ± 0.05
UF-yoghurt	22.24 ± 0.21	9.00 ± 0.11	4.26 ± 0.09	8.20 ± 0.13	0.78 ± 0.01
RO-yoghurt	23.22 ± 1.03	6.82 ± 1.48	9.07 ± 0.02	6.25 ± 0.07	1.08 0.05
Direct reconstitution yoghurt	22.50 ± 0.19	6.38 ± 0.04	8.72 ± 0.31	6.10 ± 0.14	1.30 ± 0.02

[a] Lactose was determined by difference.
(After: Ozer *et al.*, 1997).

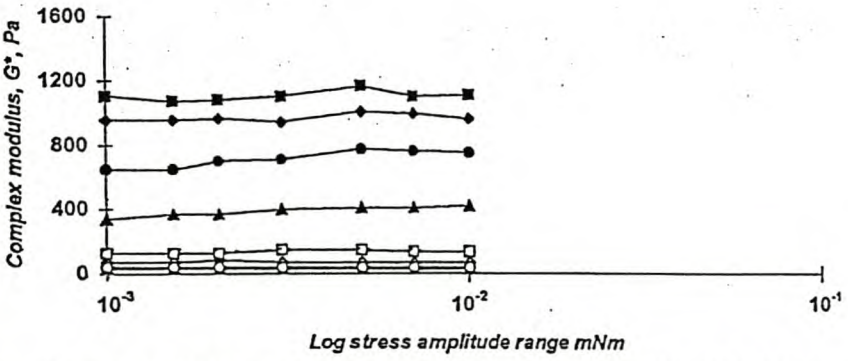

Fig. 1. Changes in the complex modulus (G^*) of the samples during incubation G^* was determined as a function of amplitude sweeps within the torque range 10^{-3}–10^{-2} mNm at 0.25 Hz. Results are the average of eight separate runs. Measuring temperature was 25°C. Measurements were taken at 120 min (open symbols) and 180 min (closed symbols) of incubation RO-milk. (△-▲), UF-milk (□-■), unconcentrated milk (◇-◆), direct reconstitution milk (○-●).

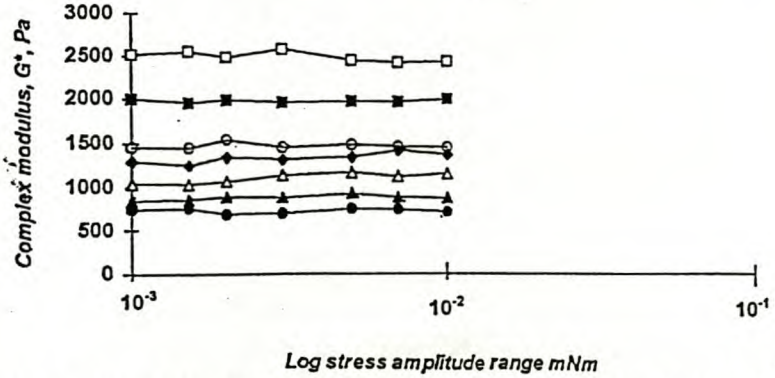

Fig. 2. Changes in the complex modulus (G^*) of the samples during incubation. G^* was determined as a function of amplitude sweeps within the torque range 10^{-3}–10^{-2} mNm at 25 Hz. Results are the average of eight separate runs. Measuring temperature was 25°C. Measurements were taken at 240 min (closed symbols) and 360 min (open symbols) of incubation. RO-milk (△-▲), UF-milk (□-■), unconcentrated milk (◇-◆), direct reconstitution milk (○-●).

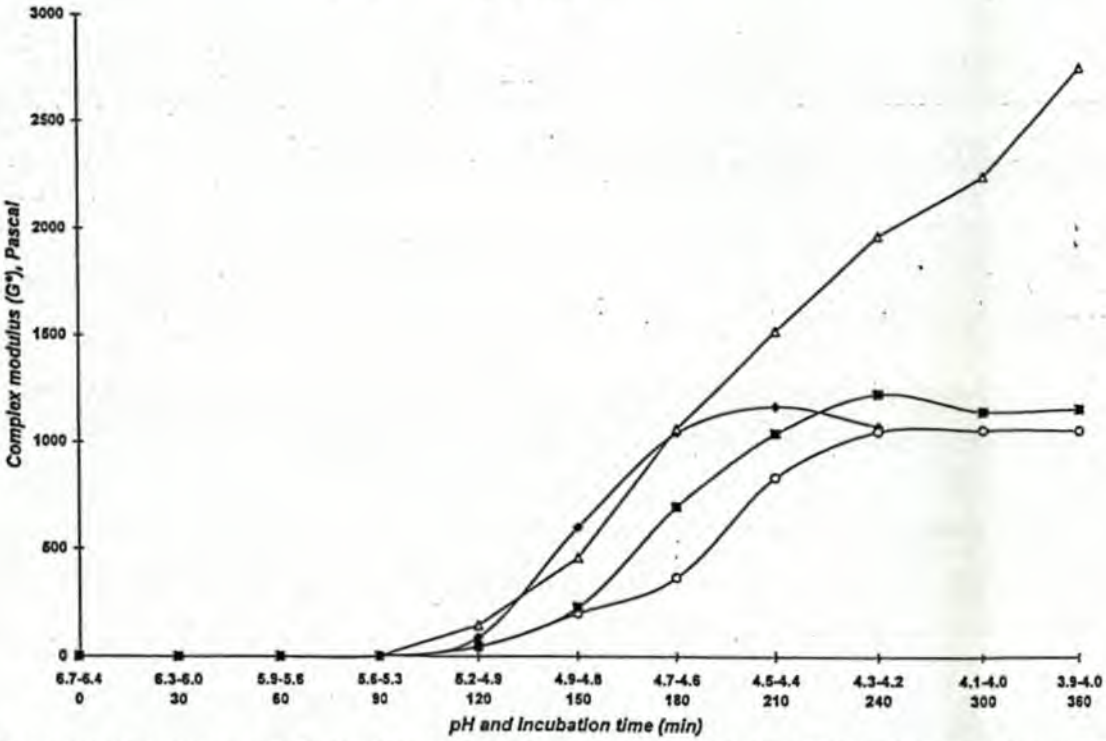

Fig. 3. Changes in the complex modulus (G^*) of the samples as funcations of incubation time and pH. Results are the average of eight separate runs. Standard errors were less than symbol dimensions. Experimental conditions: Amplitude range 10^{-3}–10^{-2} mNm at 0.25 Hz; temperature 25°C, parallel-plate geometry, plate radius 10 mm, gap setting 1 mm. Figures were derived from the centre point of the linear viscoelastic region of each sample (5×10^{-3} mNm). Unconcentrated milk (♦), direct reconstitution milk (■), RO-milk (○), UF-milk (△).

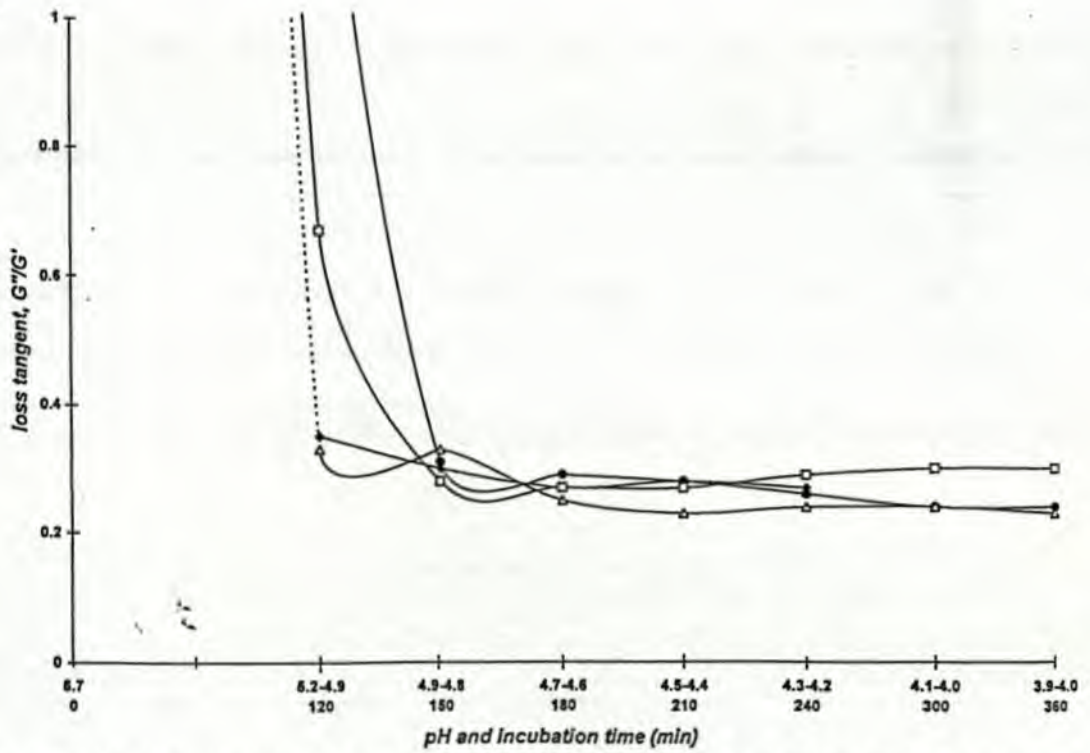

Fig. 4. Changes in the loss trangent ($\tan\delta$) values of the samples as functions of time and pH. Experimental conditions: Amplitude range 10^{-3}–10^{-2} mNm at 0.25 Hz, temperature 25°C, parallel-plate geometry, plate radius 10 mm, gap setting 1 mm. Results are the average of eight separate runs. Figures were derived from the centre of the linear viscoelastic region of each sample (5×10^{-3} mNm). unconcentrated milk (♦), direct reconstitution milk (□), RO-milk (●), UF-milk (△).

the samples, but it was not detectable, and the first positive dynamic measurements were recorded when the storage modulus coincided with an initial value of about 10 Pa.

Figures 3 and 4 show the changes in the complex modulus (G^*) and loss tangent (tan δ) values of the test samples as functions of the pH and incubation time. During the early stages of incubation (0–90 min), no viscoelasticity was evident in any of the samples but as the pH decreased, an increase in the complex modulus (G^*) was observed (Fig. 3) which was associated with a transition from the 'liquid' to the 'gel' state. This change was evident from the sudden decrease in the loss tangent (tan δ) (Fig. 4), and was similar to that observed for normal yoghurt (Biliaderis *et al.*, 1992). The 'gel' onset point for all samples was in the critical range for acid gel formation, i.e. pH 4.9–5.2 (Heertje *et al.*, 1985), and seemed to be essentially independent of the level of total solids. After the gels had formed, the loss tangent (tan δ) remained almost unchanged for each sample throughout the incubation period, suggesting the formation of essentially similar network structures.

In contrast with most of the samples, the complex modulus (G^*) of the UF-milk sample did not reach a steady 'plateau' within the experimental time-frame, but continued to increase over the entire period of incubation. The loss tangent (tan δ) values of the UF-milk were, however, similar to those observed for the other samples, and this pattern suggests that the same interactive forces were involved in gel formation in all cases, but to different degrees.

From the above data, it was possible also to calculate the apparent gel development rates of the different milks, and Table 2 shows the results, together with the gelation times (taken as the time in minutes to attain a G^* of ~10 Pa). Overall, the gel development rate was highest in the control milk (16% TS), followed by the direct reconstitution, RO and UF milks in decreasing order, i.e. the lower the level of protein, the faster the rate of gel formation. This comparison suggests that in high protein systems, like the UF-milk, flocculation kinetics may change so that, in effect, the system enters into a 'metastable' state in which the strength of the gel can continue to increase (Schulze *et al.*, 1991); the shape of the curve for

Table 2. Apparent Gelation Rate Constant ($k \times 10^{-3}$ min^{-1}) and Onset of Gelation (Time in Minute to Attain a G^* of ~10 Pa) of Concentrated Yoghurt upon Fermentation ($n = 8$)

Parameters	Unconcentrated milk (16% TS)	UF-milk (~23% TS)	RO-milk (~23% TS)	Direct rec. milk (~23% TS)
Gel development rate	18.3 ± 1.56^a	4.88 ± 0.51^b	8.66 ± 0.34^c	8.62 ± 0.70^c
Gel onset point	105 ± 4.8	90 ± 3.1	120 ± 2.3	115 ± 1.5

Samples showing a common superscript in row did not differ significantly ($P > 0.05$).

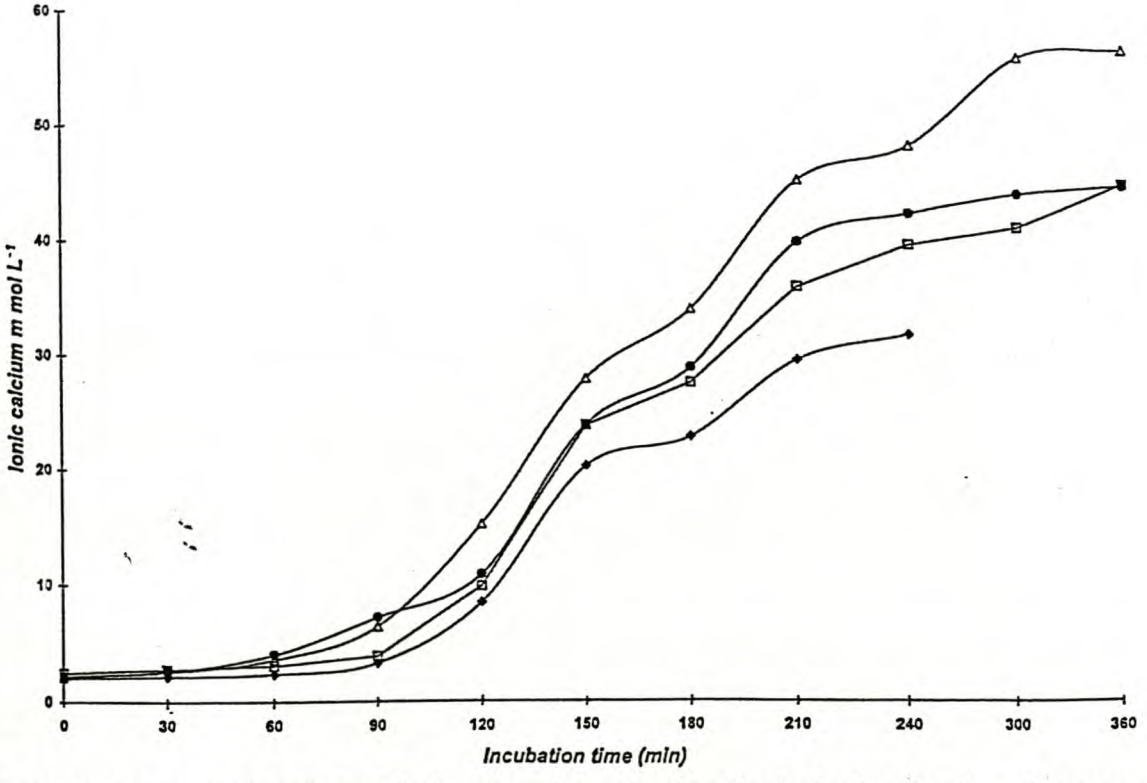

Fig. 5. Changes in the concentration of ionic calcium in test samples as a function of incubation time. Results are the average of three separate runs. Standard errors were less than symbol dimensions. Unconcentrated milk (♦), direct reconstitution milk (□), RO-milk (●), UF-milk (△).

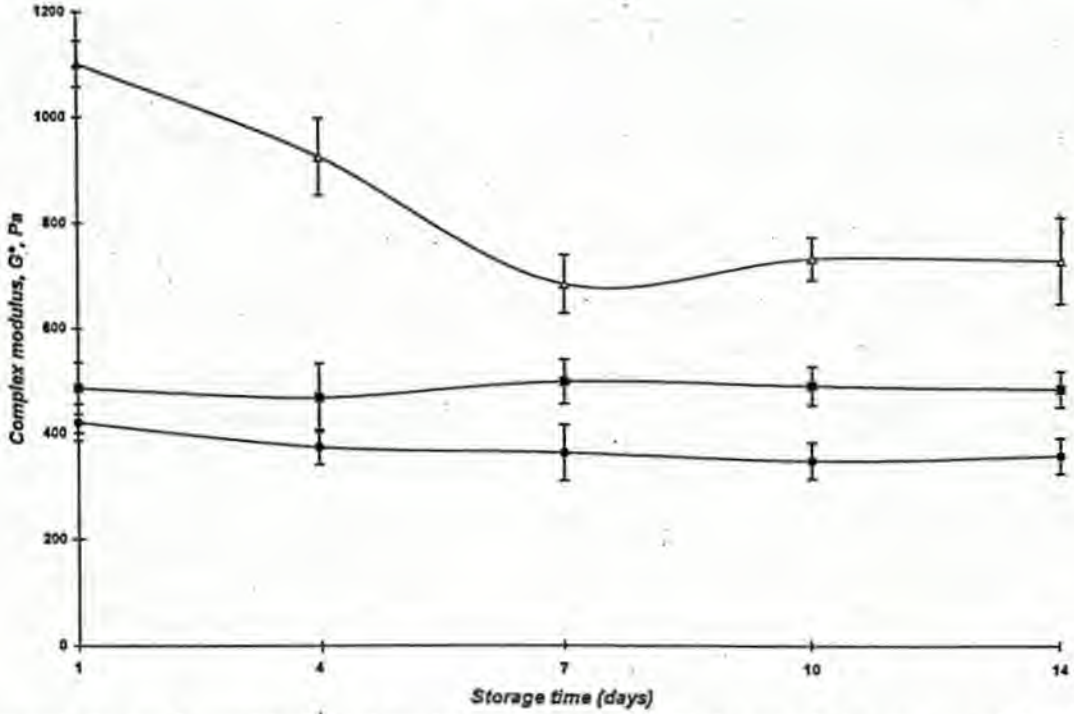

Fig. 6. Changes in the complex modulus (G^*) of samples as a function of amplitude sweep during storage period up to 14 days. Results are the mean of eight separate runs each done five times. Figures were derived from the centre point of the amplitude sweep within the linear viscoelastic region (7×10^{-2} mNm). UF yoghurt (△), direct reconstitution yoghurt (■), and RO-yoghurt (●).

the G^* data (Fig. 3) indicates that the number and/or distribution of the strong and weak bonds within the gel was increasing with time (Dickinson, 1994). The similarity of the gel onset points indicates that the rates of acidification of the milks were similar, so that once the critical pH had been achieved, gelation was observed irrespective of the level of protein present; only consolidation of the gel was dependent on the actual concentration of protein.

Changes in the concentration of ionic calcium, $[Ca^{2+}]$, as a function of incubation time are shown in Fig. 5, and the point at which the gels became visible (90–120 min, pH ~5.0) is clearly associated with an increase in $[Ca^{2+}]$; this pattern was seen in all samples. There was no apparent correlation between the $[Ca^{2+}]$ and the magnitude of the complex modulus, but the slow release of Ca^{2+} with increasing acidity is evident. Below pH ~5.0 (around 120 min), the release of ions from the coagulating protein became quite marked, providing further evidence, albeit indirect, that gelation was taking place.

According to Roefs (1986), the number of 'effective' bonds between two casein particles increases at low pH as the number of negative charges decreases, along with the magnitude of electrostatic repulsion, until a stable state exists. If this is the case, then it would appear that the rate of decrease of the repulsive forces was slower in the UF-milk than in the other samples, and that the stationary phase may not have been reached during incubation, a suggestion confirmed to some extent by the behaviour of UF-yoghurt during storage for 14 days at 4°C (Fig. 6). Thus, while the complex modulus (G^*) of the RO- and direct reconstitution yoghurts remained constant throughout the storage period, a gradual decrease in the network modulus of the UF-yoghurt was observed during the first 7 days of storage. As changes in pH throughout the storage period were negligible, it seems likely that slow protein rearrangements were continuing and more protein–protein contacts being established. Such rearrangements of the protein network are quite likely in acid casein gels (Ross-Murphy, 1990).

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The Behaviour of Starter Cultures in Concentrated Yoghurt (Labneh) Produced by Different Techniques

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Concentrated yoghurts were produced by fermenting milks concentrated by ultra-filtration and reverse osmosis, and by removing whey from batches of natural yoghurt using the same techniques. The choice of procedure influenced the chemical composition of the end-products and also, as indicated by the titratable acidities and acetaldehyde contents of the labnehs, the metabolic activity of the starter culture. Although the behaviour of the cultures varied with the total solids in the milk, the total colony counts of Streptococcus thermophilus *and* Lactobacillus delbrueckii *subsp.* bulgaricus *recorded after 24 h at 4°C were similar in all samples. A taste panel assessment revealed a definite preference for labneh (230 g/kg total solids) produced at this concentration by ultra-filtration of natural yoghurt (160 g/kg total solids).*

Keywords: concentrated yoghurt; membrane processes; starter cultures

Introduction

Concentrated yoghurt, which is popularly known as 'labneh' in the Middle East and as 'strained yoghurt' in Greece and the rest of Europe, is consumed as a main dish at breakfast in many middle eastern countries, such as Iraq, Iran and the Lebanon, but it can also be served as a dip with garlic, dried herbs (usually mint and parsley) and red peppers, or with cucumber and olive oil. Traditionally, concentrated yoghurt was made by pouring normal yoghurt (120–140 g/kg total solids) into a cloth or animal-skin bag and allowing some of the whey to drain away until the residual product had a composition of some 230–250 g/kg total solids, 80–100 g/kg fat and an acidity of 1.8–2.0 g/100 g lactic acid (1). Although the modern product has a broadly similar composition, the options for manufacture include: direct recombination of full-cream milk powder; ultra-filtration of normal yoghurt; fermentation of milk concentrated to 230 g/kg by ultra-filtration; reverse osmosis treatment of normal yoghurt; fermentation of milk concentrated to 230 g/kg by reverse osmosis; and centrifugal separation of the whey from normal yoghurt (2).

How these treatments might affect the microflora of the end product has not been subject to any systematic examination, however, and hence the aim of this project was to manufacture concentrated yoghurts by a number of different methods and then, firstly, monitor the behaviour of the microflora of starter origin; and secondly, assess the labnehs organoleptically to determine whether any microbiological changes would be reflected in the flavour or acceptability of the product.

Materials and Methods

In order to standardize the base milk, full cream milk powder supplied by Adams Food Ingredients Ltd., Leek, Staffs. U.K. was used throughout and the powder was stored at 2°C until needed. The starter culture (CH1) was obtained from Chr. Hansen's Laboratory, Reading, U.K. (Freeze-dried Redi-Set, Lot No: 5018031) and was a blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in equal proportions. This culture was selected because it generates high levels of acetaldehyde and other flavour compounds during fermentation, but little, if any, extra-cellular polysaccharide which might interfere with membrane processing.

The working culture was prepared by adding a few milligrams of freeze-dried culture to 100 mL of previously reconstituted and sterilized (121°C/2 min) skim-milk with total solids of 110 g/kg. This mixture was then incubated at 42°C until the onset of gelation. Two millilitres of culture from this first passage were transferred

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into 100 mL of sterile skim-milk at 42°C and, once again, the culture was incubated until a gel had just formed. This second culture was used for the propagation of a bulk culture (1 L) for inoculation of the different milks used for each trial. Bulk cultures were prepared 1 day before the production of yoghurt or labneh.

Full cream milk powder was reconstituted in water to 160 g/kg total solids both for making normal yoghurt and for membrane processing to 230 g/kg prior to fermentation (ultra-filtration, UF and reverse osmosis, RO, milks); the milk for labneh made by direct reconstitution was mixed to 230 g/kg total solids. In all cases, the required amount of milk powder was weighed into a sterilized stainless steel container containing the required volume of tap water at 40°C; a Silverson Emulsifier supplied by Brook Motors Ltd., Huddersfield, U.K., was used for reconstitution. A modified Vat-heating system was used to heat the milks to 85°C, and each batch was held for 20 min with constant, slow-speed stirring. Cooling was achieved in a cold water bath and all samples were cooled down to 45°C within 20 min. The reconstituted milks were then inoculated with the yoghurt culture (CH 1) at a 20 g/kg ratio and either incubated at 42°C in bulk (5 L batches) for the yoghurts to be concentrated after fermentation, or in 150 mL polyethylene cups when the total milk solids were already at 230 g/kg. The incubation of the bulk milks was halted at pH 4.3 to allow for further acidification during concentration, while the milks in cartons were held at 42°C until a final pH value of 4·0 had been reached.

A Patterson Candy pilot-scale UF plant (Patterson Candy Whitchurch, Hampshire, U.K.) was used to concentrate the milks and yoghurts. The UF cartridge consisted of a bundle of tubular membranes, the specifications of which were: surface areas, 0.8 m^2 type, ES 625; membrane material polyether sulphone and nominal molecular weight cut-off, 25,000 Da. For RO, the membrane specifications were: surface area, 1.2 m^2, type, ZF 99; and membrane material, polyether sulphone.

After the fermentation and concentration (products designated 'UF/RO labnehs') and concentration and fermentation processes (products designated 'UF/RO milks'), all samples were kept in the refrigerator at 4°C along with the traditional (cloth bag) and direct reconstitution labnehs.

Methods of analysis

The total solids levels of the samples, the pH values, using a pH-meter model Kent EIL 7045/46 fitted with a standard, combination glass electrode and the titratable acidities were determined according to the British Standard Institution Methods (3).

Lactose was determined according to the Lawrence Method (4) and the fat content by the Gerber method using Gerber (0–80 g/kg) butyrometer tubes as cited in the British Standard Institution Method (5). Total nitrogen was determined by the standard Micro-Kjeldahl procedure (5) and the crude protein value obtained by multiplication by 6.38. Acetaldehyde was measured by an enzymatic assay based on ultra-violet (UV) measurements (6).

Enumeration of yoghurt starter bacteria

A different medium (TPPY-Eriochrome Agar) developed by Bracquart (7) was used, with a slight modification, to quantify the yoghurt starters. In particular, the Eriochrome Black T dye was replaced by Prussian Blue dye to obtain better differentiation between the two genera (8). The medium was freshly prepared before each trial and the poured plates were dried at 36°C for 16 h before use. At intervals of 60 min, two samples of approximately 20 g were taken from a bulk sample or two cartons removed from the incubator in order to measure the pH, titratable acidity and total colony counts of each starter organism. Serial dilutions of the products were made in sterile peptone water (1 g/L, 9 mL) and 0.1 mL aliquots of dilutions 10^{-1}–10^{-6} were spread on the surface of duplicate plates. The Petri dishes were incubated at 36°C for 48 h under anaerobic conditions provided using Gas Generating Kits (Unipath Ltd., Basingstoke, Hants., U.K., Anaerobic System BR 038B). After incubation, the plates were removed from the jars and left for approximately 1 h under aerobic conditions to improve differentiation; the two organisms were recognized on the basis of the morphology of their colonies. *Streptococcus thermophilus* appeared as spherical colonies which were opaque white-violet with a darker centre, while *L. delbrueckii* subsp. *bulgaricus* formed larger colonies of an irregular shape, around which blue zones appeared after 1 h under aerobic conditions.

Sensory analysis

The sensory analyses were carried out according to the scheme proposed by Pearce and Heap (9). Ten panelists familiar with the product evaluated the samples after overnight storage at 5°C.

Statistical analysis

The statistical analysis of the results was completed using the Excel software programme (Microsoft Corporation, Microsoft Way, Redmond, Washington, WA 98052-6399, U.S.A.) and significantly different groups were detected by the Duncan test (10).

Results and Discussion

A typical labneh contains 220–230 g/kg total solids and has an acidity of around 1.60–1.80 g/100 g lactic acid (1). The data in **Table 1** confirm that the chemical compositions of labnehs produced by the different manufacturing techniques were similar to commercial products with respect to total solids; only the labneh made using RO concentration of the yoghurt after fermentation failed to achieve the expected compositional values. As the UF and traditional methods of concentration are selective, the fat and protein were increased in these products at

Table 1 Chemical composition of labnehs produced using different methods of manufacture

	Composition (g/100g)[c]									
	Total solids		Protein		Fat		Lactose		Acidity[d]	
Sample	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Traditional	23.3	0.24	7.45	0.11	9.18	0.11	5.66	0.07	1.7	0.01
UF labneh[a]	22.6	0.67	7.51	0.43	8.45	0.07	6.45	0.06	1.7	0.03
RO labneh[a]	20.2	0.20	6.38	0.20	5.60	0.06	7.24	0.03	1.7	0.03
UF milk[b]	22.4	0.11	7.75	0.25	8.70	0.06	5.91	0.07	2.0	0.06
RO milk[b]	23.2	1.03	6.82	0.13	6.25	0.07	8.98	0.02	1.8	0.03
Recom. labneh	22.5	0.19	6.38	0.04	6.10	0.14	8.72	0.31	1.8	0.06

After reference (11)
[a] Labnehs prepared by concentrating normal yoghurt (160 g/kg TS)
[b] Labnehs made from milks concentrated by UF and RO to approximately 230 g/kg TS
[c] Means ($\bar{x}$) and standard deviations (s_x) of duplicate samples from two separate trials
[d] Titratable acidity as 'lactic acid'

the expense of lactose and minerals, with the slow-draining cloth bag system giving the best retention of fat.

As seen from **Fig. 1**, the growth of *S. thermophilus* was similar in all samples, with the exponential growth phase ending at around 180 min of incubation. At this point, the pH values of the milks were between 5.23 and 5.60 which is probably a reflection of minor differences between the bulk samples. During the next period of incubation, a stationary phase of growth for *S. thermophilus* was observed, while *L. delbrueckii* subsp. *bulgaricus* grew more rapidly until the end of the incubation (240 min). During concentration of the yoghurts, the counts of *S. thermophilus* countinued to increase, even though the membrane treatments involved the yoghurts being circulated through the plant at 50°C. A similar, albeit more limited, rise in the counts of *L. delbrueckii* subsp. *bulgaricus* was also observed, with the more even temperature profile of the traditional system being more conducive to growth. The final acidities in all the products were similar (see **Table 1**).

In the samples of milk that had been concentrated prior to fermentation, the numbers of viable colonies of *S. thermophilus* increased rapidly up to 180 min and then remained stationary throughout the subsequent incubation and storage period (see **Fig. 2**). The final counts were below those recorded in the traditional and UF labnehs. This cessation of growth was caused by the development of lactic acid in the milks, as although the typical value at 180 min in milks of 160 g/kg total solids was 0.75 g/100 g, in the milk concentrated by UF to 230 g/kg total solids, the acidity had reached 1.1 g/100 g. The counts of *L. delbrueckii* subsp. *bulgaricus* were slightly higher than

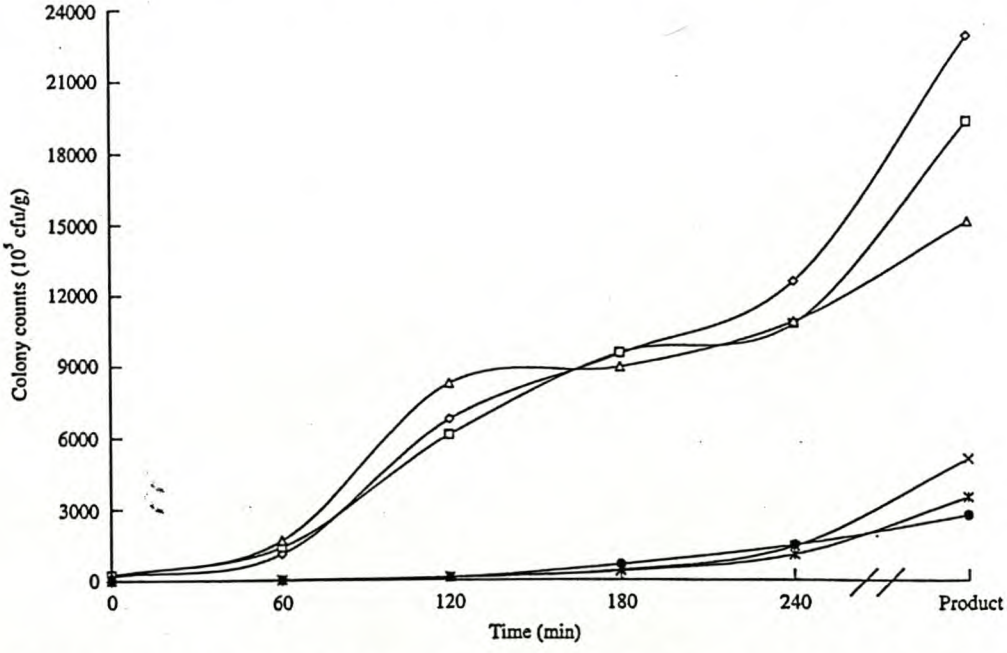

Fig. 1 Total colony counts of *S. thermophilus* (open symbols) and *L. delbrueckii* subsp. *bulgaricus* (closed symbols) during incubation of milks of 160 g/kg total solids for 240 min (pH 4.3) and in the finished labnehs (pH 4.0). All figures are shown as means of duplicate samples from two separate trials. (—◇—) = Traditional labneh, (—□—) = UF labneh, (—△—) = RO labneh, (—×—) = traditional labneh, (—*—) = UF labneh and (—●—) = RO labneh

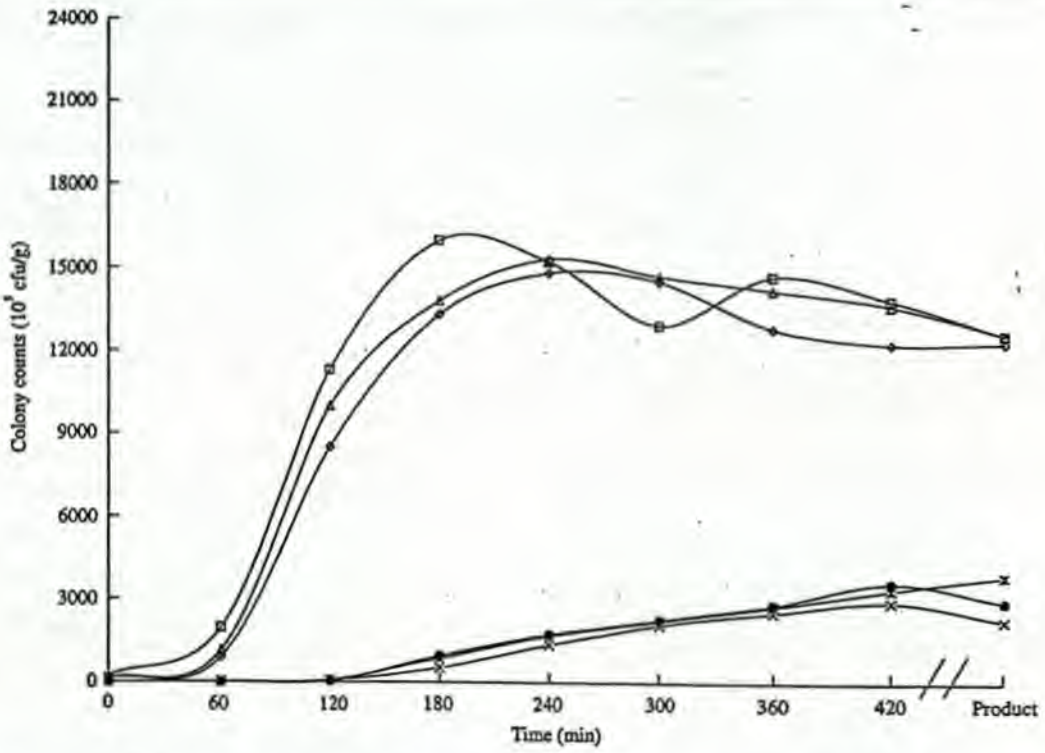

Fig. 2 Total counts for *S. thermophilus* (open symbols) and *L. delbrueckii* subsp. *bulgaricus* (closed symbols) during incubation of milks of 220–230 g/kg total solids for 420 min (pH 4.) and in the finished labnehs. All figures are shown as means of duplicate samples from two separate trials. (—×—) = Recom. milk, (—•—) = UF milk, (—●—) = RO milk, (—◇—) = Recom. milk, (—□—) = UF milk and (—△—) = RO milk

in the other milks (160 g/kg TS) after 240 min, and continued to rise throughout the incubation period. In the end products, the counts of *L. delbrueckii* subsp. *bulgaricus* were broadly similar in all the products.

The ratio of *S. thermophilus* to *L. delbrueckii* subsp. *bulgaricus* at the beginning of incubation and in the finished products varied very little between samples. Thus, initially, *S. thermophilus* contributed 94.8–96.7% of the total bacterial population in the samples, but this figure then declined in the final products to 77–85%. According to Beal and Corrieu (12), one effect of protocooperation between yoghurt bacteria is that, after the lag phase, *S. thermophilus* forms 95% of the total population, before the growth of *L. delbrueckii* subsp. *bulgaricus* reduces the dominance of *S. thermophilus* to 85%. Additionally, due to its less acid-tolerant nature, *S. thermophilus* rarely reduces the pH below 5.0, but *L. delbrueckii* subsp. *bulgaricus* is able to produce approximately three times more acid than its partner and reduce the pH to 3.8.

Similarly, Mitchell and Sandine (13) indicated that *S. thermophilus* gave higher colony numbers at every stage of incubation, even when the incubation temperature was higher than its optimum. Such observations would suggest that the symbiotic relationship between the two yoghurt bacteria tends to favour *S. thermophilus* (14), even though it should be noted that counting techiques which break up the long chains of cocci comprising 5–20 cells (15) tend to change the recorded ratio in favour of *S. thermophilus* as *L. delbrueckii* subsp. *bulgaricus* is in short chains of rods (two to three cells).

Although the total colony counts in the final products were similar, the numbers of *S. thermophilus* were highest during the exponential growth phase in UF-treated milk. This rapid development could be due to the higher concentration of stimulatory factors, such as whey proteins, retained in the UF milk (16). This effect was confirmed, to some extent, by calculations of maximum generation times (G_{max}) for the mixed cultures. In general, the cultures growing in milks with high total solids had shorter generation times (1.15–1.23 h) compared with times of 2.13–2.20 h for cultures in milks with 160 g/kg total solids.

A relationship between *S. thermophilus* and acidity was also evident and regression coefficients between counts of *S. thermophilus* and pH at the time of sampling varied from 0.829 to 0.935 in all samples. In contrast, the counts of *L. delbrueckii* subsp. *bulgaricus* were not markedly affected by acidity (r^2 between 0·536 and 0·668) in milks with lower total solids. When the total solids of the milks was elevated to approximately 230 g/kg, however, the effect of acidity on the growth of *L. delbrueckii* subsp. *bulgaricus* become relatively more important (r^2 between 0.79 and 0.845). This contrast could perhaps be attributed to the more prolonged exposure of the cells of *L. delbrueckii* subsp. *bulgaricus* to levels of acidity above 1.0 g/100 g during the longer incubation periods used with the concentrated milks. These results are consistent with those of Tramer (17) and Sinha (18) and, as might be

Table 2 Sensory properties of labnehs produced using different methods of manufacture and the levels of acetaldehyde (mg/kg) recorded in the same products

Sample	Appearance[c]		Body/texture[c]		Aroma/flavour[c]		Overall scores[d]		Acetaldehyde[c]	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Traditional	2.38*	0.91	2.09*	0.24	4.76*	0.33	9.23*	0.17	9.9*	1.00
UF labneh[a]	4.30†	0.03	4.16†	0.09	7.00†	0.06	15.46†	0.02	22.8†	1.50
RO labneh[a]	0.76¶	0.05	1.14¶	0.03	3.04‡	0.02	4.94¶	0.04	15.0‡	0.65
UF milk[b]	2.96/·‡	0.11	2.90‡	0.19	6.60†	0.09	12.19‡	0.13	15.5‡	1.60
RO milk[b]	3.12‡	0.06	3.34‡	0.03	6.24†	0.21	12.70‡	0.15	14.1‡	0.40
Recom. labneh	3.12‡	0.12	2.98‡	0.02	6.24†	0.05	12.34‡	0.07	16.1‡	0.80

[a] Labnehs prepared by concentrating normal yoghurt (160 g/kg TS)
[b] Labnehs made from milks concentrated by UF and RO to approximately 230 g/kg TS
[c] Means ($\bar{x}$) and standard deviations (s_x) of duplicate samples from two separate trials
[d] Appearance and body/texture were scored out 5 and aroma/flavour out of 10
Samples within a column showing a common superscript do not differ significantly ($P > 0.05$)

expected, this pattern was reflected in the levels of acid produced. In the milks which were concentrated and then converted to labneh, the titratable acidities in the end products were higher than in the labnehs concentrated after fermentation (see **Table 1**).

The favourable growth pattern observed in the UF concentrated milk was confirmed by the higher level of acetaldehyde (see **Table 2**), but it was notable that the taste panel were not conscious of this difference. In terms of overall acceptability, the labneh produced by UF concentration of normal yoghurt (160 g/kg TS) was the preferred option but the higher pressures associated with the RO treatment proved too destructive. This reaction of the taste panel, combined with the retention of high microbial counts of starter origin in the end product, suggests that the UF concentration of fermented milk would be the most appropriate method for the large-scale production of labneh.

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Does quantity compromise quality?

J H Al-Jedah and R K Robinson investigated the chemical and microbiological characteristics of fermented milks purchased from retail outlets in Qatar

As yogurt is believed to have originated in the Middle East, it is no surprise that even today it remains, along with dates and fish, one of the most popular of the traditional foods. Tonnage quantities of natural or flavoured set yogurts are produced locally or imported, and the concentrated yogurt labneh is an essential feature of any breakfast table.

For most consumers, it is the clean, mildly acidic taste that appeals, but it is important that the increase in milk solids in yogurt will improve the compositional analysis of yogurt vis-a-vis milk, eg >5.0% total protein versus 3.2% in full cream milk or >180mg ml^{-1} of calcium versus 115mg in milk (1).

In addition, the bioavailability of nutrients like calcium and zinc is enhanced as a result of the fermentation. Some increases in B vitamins and folic acid have been recorded as well. Furthermore, traditional yogurt has always contained 3-4% milk fat, while labneh will contain 9-10% fat, and it should not be forgotten that lipids are an integral part of any balanced diet.

Equally important may be the proposed prophylactic/therapeutic role for yogurt. Well-made products should contain, at the time of sale, millions of cells of the two cultures employed during the fermentation, namely *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*. Although neither organism is likely to survive the digestive process in humans, it is feasible to suggest that, as the cells autolyse within the small intestine, the liberated enzymes and other cell contents could have the effect of stimulating the beneficial native microfloras of *lactobacilli* and *bifidobacteria*. It is for this reason that food regulations in many countries stipulate that yogurt must, at the point of sale, have colony counts for each species in the region of 10×10^6 colony-forming units (cfu) per ml of yogurt.

Consequently, there are sound nutritional reasons for encouraging the consumption of yogurt but, as consumer demand rises, so does the risk that quality standards might be sacrificed as output increases. For example, a reduction in the level of milk solids could be used to increase the volume of yogurt produced from a given tonnage of skim milk powder, but clearly such a change would be at the expense of the nutritional value per retail unit of end-product. Again the throughput of an incubation room could be increased by fermenting the milk to a higher pH but, not only will the cell counts of the culture be lower but, at a

Photo by Nigel Kir

Table 1. Mean values of the major constituents of full cream milk and natural yogurts purchased in Qatar compared with typical yogurt samples analysed in Europe

Product constituent	Milk	Full fat yogurt					Low fat yogurt			European yogurt samples	
		A	B	C	D	E	A	B	C	Full fat	Low fat
Water (g)	87.8	85.3	87.1	84.8	86.3	86.0	86.2	90.2	88.8	81.9	84.8
Energy*	66.0	67.0	66.0	75.0	73.0	72.0	52.0	44.0	46.0	79.0	61.5
Protein (g)	3.2	4.2	3.8	4.2	3.4	3.4	4.6	2.9	3.8	5.7	5.4
Fat (g)	3.9	3.3	3.4	3.6	4.1	3.9	0.5	1.1	1.0	3.0	1.0
Ash (g)	–	0.9	0.7	1.0	0.7	0.9	1.0	0.7	0.8	–	1.2
Sugars (g)	4.8	6.2	5.9	6.4	5.5	5.8	7.7	5.1	5.5	7.8	7.6
Acidity**	–	0.8	0.8	0.7	1.0	1.0	0.9	0.8	0.7	–	–
pH***	–	4.4	4.2	4.4	4.1	4.3	4.4	4.0	4.5	–	4.0
pH****	–	4.3	4.1	4.2	4.1	4.1	4.4	4.0	4.3	3.8	3.9
Calcium (mg)	115.0	110.0	43.0	125.0	98.0	70.0	100.0	45.0	104.0	208.0	–
Sodium (mg)	55.0	55.0	41.0	80.0	58.0	50.0	51.0	33.0	90.0	80.0	–
Potassium (mg)	140.0	215.0	131.0	265.0	339.0	241.0	196.0	133.0	219.0	280.0	–

*kcal by calculation **as lactic acid ***at delivery date ****at expiry date
Letters A to E identify different manufacturers. All components are recorded per 100g

Table 2. Mean values of the major constituents of laban and labneh purchased in Qatar

Product constituent	Full fat laban			Low fat laban	Labneh		Greek-style yogurt (UK)
	A	C	D	A	A	C	
Water (g)	88.2	88.1	88.6	90.8	69.8	75.7	77.0
Energy*	61.0	64.0	59.0	39.0	205.0	143.0	115.0
Protein (g)	2.8	3.0	2.9	3.1	5.1	7.2	6.4
Fat (g)	3.3	3.8	3.1	1.0	17.0	9.6	9.1
Ash (g)	0.6	1.2	0.7	0.6	1.3	1.0	–
Sugars (g)	5.1	4.3	4.8	4.5	6.9	6.5	–
Acidity**	0.6	0.5	0.6	0.6	0.8	1.2	–
pH***	4.5	4.7	4.4	4.6	4.7	4.3	–
pH****	4.3	4.5	4.4	4.4	4.5	4.2	–
Calcium (mg)	85.0	108.0	61.0	84.0	128.0	79.0	150.0
Sodium (mg)	55.0	135.0	38.0	56.0	123.0	222.0	–
Potassium (mg)	261.0	159.0	209.0	292.0	393.0	173.0	–

*kcal by calculation **as lactic acid ***at delivery date ****at expiry date
Letters A to D identify different manufacturers. All components are recorded per 100g

pH above 4.3, there is always the risk that contaminant food-borne pathogens may survive.

There were no reasons to suppose that any of the retail yogurts on sale in Doha, the capital of Qatar, were sub-standard with respect to their chemical or microbiological properties but, nonetheless, it was decided in the interests of consumer confidence to analyse typical samples from a range of brands for levels of major nutrients and check that an abundant and viable microflora of starter origin was present at the end of the stipulated shelf life.

Five different brands of fermented milks were identified in the retail food outlets located around the centre of Doha. Three brands (A, B, D) were imported from neighbouring countries in the Gulf, while a further two manufacturers (C, E) were based locally.

Each manufacturer produces the most widely consumed type of yogurt – full fat, natural, set yogurt – but the low fat variety is available only from one local (C) and two external suppliers (A, B).

Another popular fermented milk is laban, the local name for buttermilk. However, as most producers tend to employ a yogurt culture of *Str thermophilus* and *L delbrueckii* subsp *bulgaricus* rather than the traditional buttermilk culture of *Lactococcus lactis* subsp *lactis*, *Lactococcus lactis* subsp *cremoris* and *Leuconostoc mesenteroides* subsp *cremoris*, laban may be better regarded as a drinking yogurt. Three suppliers (A, C, D) make full fat laban; only producer A makes a low fat product.

One local manufacturer (C) produces concentrated yogurt, or labneh, and there is one imported brand (A). The dearth of suppliers is surprising given the popularity of labneh. Whether or not the cost of disposing of the labneh whey is a limiting factor is not clear (2), but certainly the choice of brands is severely limited.

For the analyses, newly-delivered retail containers of the various products were purchased from local outlets and transported to the laboratory in a cool box containing ice. On arrival, duplicate containers of each product were taken for immediate microbiological analysis, two containers were taken for chemical analysis and a further two were stored at 4°C for microbiological testing at the end of the expiry date.

Two containers of any given product were macerated in a high-speed blender and duplicate sub-samples (50g) of the macerate were extracted for analysis for moisture, crude protein, fat and ash. The moisture and fat contents of each sub-sample were determined according to the British Standards Institution (BSI) method (3), and the ash content according to the Agricultural Development and Advisory Service (ADAS) (4).

The total nitrogen content of the sample was found by the Dumas method using a Leco Nitrogen Determinator; the protein content was calculated as total nitrogen multiplied by 6.38 (5).

Total carbohydrates were estimated by difference, and the energy value was calculated using the formula suggested by Kirk and Sawyer (5). The pH measurements were carried out using a Pye Unicam Ph meter (model 290 MKII).

For determination of the individual minerals, the ash was digested in 5ml of 2N HNO_3 (Analar Grade) by boiling for about two minutes and then cooling to room temperature. The cooled solution was filtered through Whatman filter paper (No 41) and made up to 25ml with 2N nitric acid. A 'blank' was prepared in a similar manner. The samples were then analysed for calcium, sodium and potassium by atomic absorption (6).

After thorough stirring, a sub-sample (10ml) was withdrawn from each container with a sterile pipette and dispensed into 90ml of sterile maximum recovery diluent (Unipath, Code No CM733) contained in a medical flat (250ml). After shaking to disperse the yogurt, further decimal dilutions down to 10^{-8} were made in 9ml amounts of the same diluent.

1ml aliquots from dilutions 10^{-5} to 10^{-8} were then dispensed, in turn, into each of four Petri dishes, and while tempered (45°C) M17 Agar (Code No CM785) was poured into two of the dishes, acidified MRS Agar (Code No CM361) was poured into the comparable pair of dishes at each dilution.

Incubation at 37°C for 72 hours in a Nuaire DH Autoflow incubator (10% CO_2) was sufficient to produce visible colonies and, after counting, Gram staining and microscopic examination of a random selection of colonies, confirmed that the colonies on M17 Agar were predominantly *Str thermophilus*, and those on the MRS

Table 3. Mean total colony counts (cfu/ml) of the starter bacteria employed to manufacture the products indicated

Product sample	*Streptococcus thermophilus*		*Lactobacillus delbrueckii* subsp *bulgaricus*		
	Delivery date	Expiry date	Delivery date	Expiry date	Shelf life
Full fat yogurt					
Brand A	2.0×10^9	1.9×10^9	1.2×10^9	1.2×10^9	10 days
Brand B	9.5×10^8	8.0×10^8	9.5×10^8	4.0×10^8	10 days
Brand C	1.8×10^9	1.9×10^9	8.5×10^8	1.4×10^9	8 days
Brand D	5.2×10^8	4.3×10^8	3.5×10^8	3.0×10^8	7 days
Brand E	1.7×10^9	1.0×10^8	2.0×10^9	1.2×10^8	10 days
Low fat yogurt					
Brand A	1.9×10^9	1.9×10^9	8.0×10^8	9.0×10^8	10 days
Brand B	6.5×10^8	6.5×10^8	9.0×10^8	1.3×10^8	10 days
Brand C	2.4×10^9	1.7×10^9	3.7×10^8	1.5×10^9	8 days
Full fat laban					
Brand A	7.0×10^8	1.1×10^9	2.6×10^7	3.4×10^7	7 days
Brand C	6.2×10^8	3.0×10^8	9.0×10^8	7.0×10^8	7 days
Brand D	2.3×10^9	1.6×10^9	2.5×10^6	1.4×10^6	7 days
Low fat laban					
Brand A	5.0×10^8	6.4×10^8	4.5×10^6	5.0×10^6	7 days
Labneh					
Brand A	2.2×10^9	2.1×10^9	1.3×10^7	1.2×10^7	10 days
Brand C	1.1×10^9	2.2×10^9	6.0×10^8	8.0×10^8	15 days

plates were *L delbrueckii* subsp *bulgaricus*. All counts were recorded per ml of product, and the procedure was repeated on the stipulated expiry date.

Nutrient profile

As indicated, one of the reasons for encouraging consumption of yogurt in Europe is that its nutrient profile is better than that of liquid milk and, furthermore, vulnerable groups like children and the elderly often find the flavour and mouthfeel of yogurt appealing. This view is supported by the analyses of a premium natural, full fat yogurt (1) (see Table 1) and a standard low fat product (7), for the levels of essential nutrients like protein and calcium are well above those associated with a typical sample of bovine milk (1). The contrast with the brands purchased in Qatar was, therefore, somewhat unexpected, particularly as the protein contents in samples D and E are not far above that in normal milk.

The levels of total solids (~14-15%) for most of the brands are fairly typical, and the protein levels suggest that some degree of fortification of the non-fat milk solids has taken place. The same is true for the low fat varieties from factories A and C, and the protein value of 4.6% in brand A is well above that in liquid milk. However, while brand B appears rather deficient in comparison with its European counterpart, it is important to record that some types of natural yogurt are sold at a low price for cooking purposes.

Nevertheless, all the yogurts meet the minimum legal standards for non-fat milk solids set in most countries at 8.2%, while the fat contents of the full fat yogurts are above the expected minimum value of 3.0%. The usual standard for the fat content of yogurt designated as low fat is <1.0% and the deliberate retention of around 1.0% fat may be a reflection of the system employed for standardisation (1).

As laban was conceived as a refreshing drink, the total solids of 11-12% (see Table 2) is probably fairly typical, even though it means that the nutritional value of the products is below that of liquid milk on sale in Europe. The high sodium level in brand C could indicate that sodium chloride has been added to enhance the consumer appeal of the product, but there could be a preservative benefit as well. Thus, a pH of 4.5 is borderline with respect to the survival of pathogens like *Listeria monocytogenes* (8) and, although the species is salt-tolerant, the combined impact of acidity and salt could prove effective should contamination ever occur (9).

The product manufactured locally by factory C appears to be a good example of commercial labneh and it is likely that its physical properties would match the best of the commercial concentrated or Greek-style yogurts (see Table 2). In particular, the fat content is above the legal standards for commercial labneh set in countries such as Saudi Arabia (7.0%) and Jordan (9.0%) (1). The low figure for calcium suggests that the original yogurt may have been concentrated by ultra-filtration, while the high sodium content could reflect the traditional practice of salting the labneh during final mixing and packaging. The acidity at 1.2% as lactic acid is lower than many traditional products which have acidities of 1.8-2.0% (1) but, as the product is made locally, it may be that the market demands a less acid taste.

The imported labneh (A) is unusual in having a higher than average level of total solids, but a protein content below that of a standard European yogurt. Similarly, the fat content is exceptionally high – even yogurt cheese with a total solids of ~40% has a fat content of only 18-19% fat (9). The titratable acidity at 0.8% lactic acid is also somewhat unexpected, especially as the high fat content would effectively mask any excessively acidic taste.

However, if the nutritional value of some of the yogurts is slightly lower than might be anticipated on the basis of the literature values, the microflora of starter origin meets with ease the designation that it should be 'abundant and viable' at the time of consumption. The balance in favour of *Str thermophilus* for the brands of laban probably reflects the modest pH (~ pH 4.5) of the products at the time of delivery, for this figure implies that the fermentation was halted by cooling the laban at around pH 4.7. *L delbrueckii* subsp *bulgaricus* is well represented, but it is important that the cell counts are not excessive in products like laban, which have insufficient total solids to mask any over-acidification.

In products incubated to give a final pH of 4.1-4.2, however, *Str thermophilus* enters a stationary phase before the fermentation is complete, and the numbers of *L delbrueckii* subsp *bulgaricus* equal or exceed the counts for *Str thermophilus*, even though the shelf lives of such products are shorter than their European counterparts.

Overall, it is evident that the counts of the microfloras of starter origin are most satisfactory and, if the release of enzymes and other components of cells autolysing in the human intestine does indeed have a beneficial effect, then the cell counts in the fermented milks from Qatar are to be welcomed.

Similarly, it is of note that all the products meet the compositional standards expected for the type of yogurt in question, so that the apparent differences between the European and local brands may simply reflect consumer preferences. DII

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Production of acetic and propionic acids from labneh whey by fermentation with propionibacteria

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Whey produced during the manufacture of labneh was supplemented with yeast extract (10 g/l), and then fortified with lactose, treated with β-galactosidase or fermented with Lactobacillus helveticus, prior to inoculation with free living cells of Propionibacterium freudenreichii ssp shermanii or Propionibacterium acidipropionici or cells immobilized in aliginate beads. Under anaerobic batch conditions, fermentation of the whey with Lb helveticus followed by P acidipropionici (free cell system) for 2.5 days at 32°C gave a broth with 5.9 g/l of propionic acid and 2.4 g/l of acetic acid, while immobilized cells of the same organisms gave a broth with 11.0 g/l propionic acid and 3.2 g/l acetic acid over 4 days. These latter values were the maximum levels recorded with any of the treatments, and it is suggested that such yields might make recovery economically feasible in certain countries.

Introduction

Labneh is manufactured from a standard natural yogurt, either mechanically[1] or by the traditional process of pouring the yogurt into a cloth bag and allowing the whey to drain slowly overnight. Irrespective of the method, the finished product has a total solids of 23–24%,[2] so that around 50% of the water in the original yogurt base is lost as whey.

In composition, labneh whey is similar to the acid whey produced by the cheese industry, except that the lactose concentration is lower following the more active fermentation by the thermophilic bacteria in yogurt. In industrialized countries the most popular method for handling cheese whey is concentration and spray drying, although alternatives such as fermentation to ethanol or lactic acid have been considered.[3]

However, less developed countries may well be inclined to encourage unorthodox processes that will eliminate a demand for foreign currency. For example, Jordan imports 60 tonnes of propionic acid and 24.3 tonnes of acetic acid per year for use within the food industry, and yet substrates for fermentation are readily available. If the production of food-grade acids by fermentation could be established as economically viable, then these materials might prove attractive in the marketplace; (a) they could find immediate application as food preservatives, and (b) their origin as fermentation products should enable them to be regarded as 'safe'.

Consequently, the aim of this project was to consider those factors that might influence the production of acetic and propionic acids from labneh whey by fermentation by *Propionibacterium freudenreichii* sp *shermanii* (*P shermanii*) or *Propionibacterium acidipropionici* and, in particular, to assess the influence of different sources of energy—namely, lactose, glucose/galactose and lactate produced by prefermentation of the whey with *Lactobacillus helveticus*—on the yields of organic acids liberated during batch fermentation. In addition, Tyagi *et al*[4] suggested that immobilized cells undergo less growth than free living cells, so that more energy is diverted to cell metabolism and, hence, product yield is higher. The studies of Champagne *et al*[5] and Begin *et al*[6] with *P shermanii* tend to support this view, so that the second stage of the project involved a comparison of the performances of free living and immobilized cells of *P shermanii* or *P acidipropionici*.

Materials and methods

Nature of the cultures

Isolates of *P shermanii* (NCFB 853) and *P acidipropionici* (NCFB 563) were obtained from the National Collection of Food Bacteria (Institute of Food Research, Reading, Berkshire RG6 2AP) and a culture of *Lb helveticus* (ATCC 15009) from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852-1776). In a positive-pressure glove-box swept with sterile, oxygen free nitrogen, the individual ampoules of the above species were resuscitated according to the instructions of Holdeman *et al*.[7]

The rehydrated cells of the propionibacteria were then transferred to tubes of Sodium Lactate Yeast Extract Broth (SLYEB) (50 ml) as recommended,[8] and the cell counts on SLYE Agar after 24 hours (anaerobic incubation at 30°C) were found to be 1.58×10^9 colony forming units/ml and 1.45×10^9 cfu/ml for *P shermanii* and *P acidipropionici* respectively. Champagne *et al*[5] suggested that such cell densities at 24 hours were likely to indicate balanced exponential growth and, consequently, all fresh cultures employed to inoculate the fermentation vessels were incubated for 24 hours.

Typical colonies on the plates of SLYEA were also examined under an Olympus microscope (Olympus Optical Company, London

Original paper.
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EC1Y 0TX) and for catalase production; the presence of pleomorphic, Gram positive, catalase positive rods was taken as confirmation that the colonies were propionibacteria.

The resuscitation of *Lb helveticus* followed a similar pattern, except that batches of De Man, Rogosa and Sharpe (MRS) broth (50 ml) were employed as the medium, and 37°C was used for incubation. Stock cultures of *Propionibacterium* were maintained as stab-cultures in SLYEA, and slopes of MRS agar were employed for routine maintenance of *Lb helveticus*; after 24 hours' incubation, the cultures were stored at 4°C.

For immobilization, bulk cultures (1 l) of *P shermanii* and *P acidipropionici* were incubated at 30°C for 30 hours in anaerobic bottles flushed with nitrogen, and the cultures were then transferred to a cabinet at 4°C for the cells to settle over 16 hours. At this point, around 900 ml of the supernatant was discarded, and the cell deposit was recovered into the remaining liquid, which was adjusted to 100 ml. An equal volume of sodium alginate solution (2% w/v) was mixed with the cell suspension, and the mixture added as single drops from a syringe into a solution of $CaCl_2$ (0.05 M). The drops solidified on contact with the $CaCl_2$ to form beads of visually uniform dimensions, and these were allowed to harden for 1 hour. The excess $CaCl_2$ was then replaced with sterile peptone water (100 ml, 0.1%), and the beads stored at 4°C until used.

Conditions of fermentation

A bulk sample of labneh whey (100 l) was collected from the dairy plant of the Faculty of Agriculture, University of Jordan, and pasteurized at 70°C for 2 minutes. It was then distributed into wide-mouth, plastic bottles of 2 l capacity for storage at −20°C.[9] Prior to use in the experimental programme, two typical bottles of whey were thawed, the contents mixed thoroughly and subsamples from each bottle analysed for total lipids according to the method of Klupsch,[10] total nitrogen[11] and lactose/total reducing sugars.[12] The lactic acid content was measured by the Mattson method[13] and pH with a Kent EIL 7020 meter and combination electrode.

For preparation of the fermentation broths, the pH of a bulk sample of thawed whey (5 l) was first adjusted to 8.5 with 2N-NaOH. This alkaline material was then heated to 95°C for 30 minutes, cooled to 5°C and held for 1 hour to allow the solids to settle; the whey was then filtered (Whatman No. 1 Paper) under vacuum. Yeast extract (1% w/v) (Fisons, Poole, Dorset) was added, and the pH adjusted to 7.1–7.2 with 2N HCl.

In an attempt to evaluate the effect of different factors on acid production, four whey samples (each of 1 l) were treated as follows: (a) the lactose concentrations were increased by the addition of 70 or 140 g/l lactose; (b) the lactose was hydrolysed with β-galactosidase[14] to give >90% breakdown; and (c) the influence of high levels of lactate was studied by inoculating (2% v/v) whey with *Lb helveticus* and incubating at 44°C for 6 hours with the pH held at 5.9.[5]

After dispensing duplicate portions (350 ml) of each broth—four treatments and a control—into 500 ml Duran® bottles, the whey was 'sterilized' at 95°C for 25 minutes.[13] The cooled broths were then inoculated with the species of *Propionibacterium* at a rate of 3% v/v (fresh culture or a well shaken suspension of alginate beads), and the bottles transferred to a shaking water bath (La. Line Instruments, USA) maintained at 32°C, with an agitation rate of 100 cycles per minute. The screw-cap of each bottle was modified to incorporate a sampling port alongside gas inlet and outlet tubes, and anaerobic conditions were maintained by gently bubbling ultra-pure, sterile nitrogen through each bottle.[7] The pH in the medium was held at around 7.0 by periodic testing and the addition, as required, of 2N-NaOH.

Sampling and analysis

Samples (10 ml) were withdrawn every 12 hours up to 60 hours (free living cells) and 96 hours (immobilized cells) to monitor: (a) lactic acid; and (b) concentrations of propionic and acetic acids by gas chromatography (GC). A Hewlet Packard Model 5890 A chromatograph was used, and the column was packed with XE-60 Silicon loaded with 3% Chromosorb WHP (Supelco, Bellefonte PA 16820-0048). The column was operated at 200°C, the detector temperature was 220°C and the carrier gas was nitrogen with a flow rate of 30 ml/min. Identification involved a Flame Ionization Detector and LCI-100 Perkin Elmer Integrator. The samples for analysis were centrifuged to remove the cells, and the supernatants filtered through a 0.2 μm membrane. The filtrates were then extracted with diethylether for injection into the GC[16]; the acetic and propionic acid standards were of GC grade.

In addition, duplicate samples were taken for microbiological analysis. Serial dilutions in sterile peptone water (9 ml, 0.1%) were prepared, and total colony counts of propionibacteria were made on pour plates of SLYEA; anaerobic incubation at 30°C for 48 hours was standard. The entire trial, including free and immobilized cells, the four treatments and the control, was repeated four times. The results were analysed using the General Linear Models procedure of SAS.[17]

Results and discussion

The analysis of labneh whey as drawn from the dairy plant was: fat 0.27 ± 0.1%, protein 0.89% (equivalent to 0.14 ± 0.009 total nitrogen), lactose 2.5 ± 0.31%, lactic acid 0.88 ± 0.02% and pH 3.6 ± 0.12.

The growth of *P shermanii* and *P acidipropionici* in the untreated whey (control) showed that there was no significant ($p > .05$) difference between the behaviour of the two species. However, there was a significant ($p > .05$) difference between the behaviour of the free living and immobilized cells of the two species with respect to the production of propionic and acetic acids (see Table).

When the level of lactose was raised by approximately 7%, the viable cell counts of both species increased significantly ($p < .05$), but the increase in the propionic and acetic acid concentrations produced by both free living and immobilized cells of *P acidipropionici* was marginal. This contrast implies that growth and acid production may be uncoupled. The addition of 14% lactose produced total inhibition of cell growth rather like that described by Wiharm and Sack[18] for yeasts, and acid production was negligible.

The effects of further modifications of the labneh whey are shown in the Table, and the influence of lactate on organic acid production was significant ($p < .05$). *P acidipropionici* reacted more favourably than *P shermanii*, but overall it was evident that the two stage fermentation had almost doubled the concentration of propionic acid in the spent medium. The work of El-Hagarawy *et al*[19] on Swiss cheeses supports the conclusion that lactate is metabolized preferentially by propionibacteria under anaerobic conditions, and Hattinga and Reinbold[20] reached a similar conclusion. It was not established whether or not the growth of *Lb helveticus* improved the nutrient status of the medium, in addition to producing lactate, but hydrolysis of the lactose did lead to higher levels of propionic acid ($p < .05$). Furthermore, Champagne *et al*[5] suggested that this fermentation is sensitive to substrate composition, and the different responses of the species to the various treatments suggests that species/strain selection could be critical as well.

Assuming that the free cell and immobilized counts were not markedly different (the alginate made the counts unreliable), then the differences in acid production, especially in the presence of lactate, are highly significant ($p < .05$). The most likely explanation of this effect is that the diffusion of lactic acid away from the cells is impeded by the alginate, and hence lactic acid is metabolized rather than lost into the medium. Obviously, the availability of lactose may be limited by the structure of the beads, but, as lactate is used preferentially by this genus, the restriction may not be important; Marcoux *et al*[21] suggested that this effect stems from the more rapid assimilation of lactate vis-à-vis lactose and its direct conversion to pyruvate.

Overall, it is evident that the treatment of labneh whey with β-galactosidase or prior fermentation by *Lb helveticus* raised the levels of propionic and acetic acids produced by *P acidipropionici* to values significantly above those in the control broth, and immobilization of the cells resulted in the concentration of propionic acid being almost doubled to 11.0 g/l in the high lactate substrate. Clearly, the economic feasibility of fermenting labneh whey to organic acids would have to be considered with respect to any given location, as would the possibility of further increasing the final concentration of the acids.

Mean (±SE) compositions of labneh whey (supplemented with 1% yeast extract) and (A) treated with β-galactosidase (90% hydrolysis of lactose), (B) fermented with *Lb helveticus* to give 3.2 g/l lactate or (C) with 7% added lactose, following fermentation by free living[1] or immobilized cells[2] by *P shermanii* (NCFB 853) and *P acidipropionici* (NCFB 563) for 60 and 96 hours respectively

Components	Control	B Lactate whey	A Hydrolysed whey	C Whey + lactose
P shermanni				
Propionic acid[1]	2.0 ± 0.03	5.6 ± 0.032	3.5 ± 0.03	2.6 ± 0.02
Acetic acid[1]	0.7 ± 0.08	2.3 ± 0.098	1.5 ± 0.09	0.7 ± 0.09
Propionic acid[2]	8.1 ± 0.02	10.0 ± 0.03	9.3 ± 0.02	7.1 ± 0.03
Acetic acid[2]	3.1 ± 0.08	3.8 ± 0.088	3.5 ± 0.09	3.2 ± 0.09
P acidipropionici				
Propionic acid[1]	2.3 ± 0.032	5.9 ± 0.035	3.8 ± 0.031	3.1 ± 0.028
Acetic acid[1]	0.7 ± 0.08	2.4 ± 0.098	1.5 ± 0.90	0.8 ± 0.079
Propionic acid[2]	7.8 ± 0.02	11.0 ± 0.03	8.5 ± 0.02	8.1 ± 0.02
Acetic acid[2]	3.0 ± 0.09	3.2 ± 0.08	3.1 ± 0.09	3.3 ± 0.089

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PRODUCTION OF ACETONE AND BUTANOL BY FERMENTATION OF LABNEH WHEY

By

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SUMMARY

Whey produced during the manufacture of labneh was supplemented with yeast extract (5 g l^{-1}) and lactose (32 g l^{-1}) prior to inoculation with *Clostridium acetobutylicum.* Under anaerobic conditions, fermentation over 7 days at 30°C gave a broth with 13.6gl^{-1} butanol, 1.6 gl^{-1} acetone and trace amounts of ethanol, organic acids were produced also. It is suggested that this level of solvents would make recovery economically feasible, and that the process could be of interest to countries seeking to avoid reliance on oil-based processes.

Key words: Labneh whey, fermentation, acetone, butanol, *Clostridium acetobutylicum.*

INTRODUCTION

Labneh is a concentrated yoghurt (total solids of 23-24%) produced widely throughout the Middle East; along the shores of the Mediterranean, a similar product is known as strained or Greek-style yoghurt. It is manufactured by removing around 50% of the liquid (whey) from a standard natural yoghurt by centrifugation (Robinson & Tamime, 1993), membrane processing (Tamime *et al.*, 1991) or by the traditional process of pouring the yoghurt into a cloth bag and allowing the whey to drain slowly overnight.

As with cheese whey (Tamime, 1993), the Biological Oxygen Demand of labneh whey is too high for casual disposal into rivers, and direct use as a fertiliser or animal feed can pose problems. Conventional waste disposal systeme are employed by some dairies for handling large volumes of cheese whey, but the most popular method is concentration and spray-drying. Alternatives, such as fermentation to ethanol or lactic acid, have been considered (Zadow, 1994,) but industrialised countries find that the

chemical industry can still produce these end-products more cheaply.

However, many countries are committed to limiting, as far as possible, their dependence on imported materials and, as current solvents production is based on oil, intterest in the fermentation of locally-available feedstocks remains active. At present, factories manufacturing labneh in the Middle East are small compared with a typical cheese plant but, if a number of producers could co-operate and agree to handle the whey at a central location,an industrial option for disposal could become feasible. One such option could be the production of solvents like butanol and acetone (Ennis& Maddox, 1987; Maddox,1980), followed by concentration and drying of the residual lactose, whey proteins and microbial biomass. If both the solvents and crude whey powder could find a local market, then the disposal of labneh whey by fermentation could become a profitable enterprise.

This interest in the butanol: acetone fermentation is based upon the facts that: (a) there is a demand for butanol in the Middle East; and (b) while many fermentations benefit from high levels of carbohydrate in the medium, maximum solvent production by *Clostridium acetobutylicum* can be achieved at carbohydrate levels not far above those found in whey (welsh& Veliky, 1984). However, a number of factors control the balance between acidogenesis and solventogenesis for any given culture of *C. acetobutylicum,* and particular stress has been laid upon the effect of nutrient limitation (Terracciano & Kashket,1986).

Therefore, given that whey is not a nutrient-rich substrate, the aim of this project was to consider the influence of carbon/nitrogen levels on the production of butanol, and to define the optimum conditions for maximum solvent yield.

MATERIALS AND METHODS

The culture:

A strain of *C. acetobutylicum* (NCIMB 619) was obtained from the National Collection of Industrial an Marine Bacteria, 23 St. Machar Drive, Aberdeen, Scotland AB2 1RY and in a positive-pressure glove-box swept with sterile, oxygen-free nitrogen, an individual ampoule of the above strain was resuscitated according the instructions of the supplier. The rehydrated cells were then surface-plated onto fresh Nutrient Yeast Glucose Agar (NYGA) as recommended by Holdeman (1977) and Alder and Crow (1987). The plates were incubated at 37°C for 4 days in polycarbonate jars containing 1.81 of hydrogen and 350 ml of carbon dioxide generated by a gas pack from Oxiod Ltd., Basingstoke, Hampshire RG24 0PW (UK).

According to Alder and Crow (1987), colonies of *C. acetobutylicum* on NYGA that have brown centres and numerous spores (designated as A-C Type I colonies) are likely to be solventogenic, and hence spore counts of brown-centred colonies were carried out using the procedure of Seeley and Van Demark (1981); plates containing typical A-C Type I colonies were sealed to prevent dehydration and stored at 4 °C.

The solvent-producing ability of typical A-C Type I cultures was tested as follows. *C. acetobutylicum* was grown on NYGA, and spores (1.2 X 10^6 ml^{-1} of inoculum) were inoculated into 100 ml amounts of sterile Potato Dextrose Medium (PDM). If, after incubation at 37°C for 7 days, the medium contained around 6.5 g l^{-1} of butanol, 1.5g l^{-1} of acetone and 0.1 g l^{-1} of total acids, the isolate was classified as A-C Type I in terms of solventogenesis.

Preparation of the Inoculum:

Spores from typical A-C Type I colonies were used to inoculate 5 ml volumes of Robertson's Cooked Meat Medium (RCMM) (Ennis & Maddox, 1985); Oxoid Manual, 1995) supplemented with glucose (10 g l^{-1}) and, after anaerobic incubation at 37°C for 24 hours, 1ml, portions were transferred to 9 ml volumes of RCMM supplemented with lactose (10 g l^{-1}). A further period (48 hours) of incubation at 37°C provided a cell suspension of sufficient optical density-visual determination only, for the culture to be used as an inoculum for labneh whey.

Portions (100 ml) of filtered whey were autoclaved at 110°C for 20 minutes in screwcap Duran bottles (100 ml capacity) - the absence of an appreciable head-space encouraged the removal of oxygen from the medium. cooled to 37°C, and inoculated with the RCMM culture at a rate of 10% v/v: the viability of the inoculum was checked by the method of Jones *et al.*, (1982). Incubation at 37°C for 48 hours gave a 'mother culture' suitable for inoculation of the larger volumes of labneh whey.

Handling of the Bulk whey:

The labneh whey (100 l batches was collected from the dairy plant of the Faculty of Agriculture, University of Jordan, and pasteurised in bulk at 70 °C for 2 minutes. It was then distributed into wide-mouth, plastic bottles of 2l capacity for storage at -20 °C (Vienne & Stockan, 1984). Prior to use, the whey from two bottles was analysed for total lipids, total nitrogen, lactose and ash and total solids according to the methods cited by Haddadin *et al.*, (1996). The lactic acid content was measured by the Mattson Method (1965) and pH with a Kent EIL 7020 meter and combination electrode.

When required for fermentation, visible fat was removed from the surfaces of the frozen samples, and the bottles placed at 4°C overnight. After coarse filtration through Watman No. 1

filter paper, the whey was heated to 40°C and the pH adjusted to 4.6 with 3N NH_4OH. One drop of silicon anti-foam was added and, following heating of the sample to 90 °C, the pH was raised to 7.5. The neutralised whey was boiled for 10 minutes and, after cooling, the denatured protein was removed by centrifugation at 1050 G for 20 minutes. The supernatants were bulked, adjusted to pH 5.0 with 1N HCl and fortified - on the basis of some preliminary trials - at a rate of 5 g l^{-1} with yeast extract powder (Fisons, Poole, Dorset. UK) - control sample.

In an attempt to maximise solvent production by altering the availability of carbon and nitrogen, some of the bulk samples were treated with β- galactosidase (Anon., 1996) to give > 90% hydrolysis; others were modified, prior to denaturation of the protein, with pepsin to determine whether amino-nitrogen would stimulate solventogenesis; while others were fortified with lactose to give concentrations of 6.5 and 9.5% w/v.

Both the modified and unmodified wheys were then dispensed as 180 ml portions into screw - cap Duran bottles (250 ml), and autoclaved at 110° for 20 minutes, cooled to 30 °C, and inoculated immediately at a rate of 10% v/v. All manipulations were completed in an atmosphere of sterile, oxygen-free nitrogen.

Methods of analysis:

Standard portions (180ml) of medium were used for all experiments, and samples (10 ml) were withdrawn daily to monitor various parameters over a period of 7 days; at this point, the pH of the medium had stabilized, and this stability was taken to indicate the end-point of solvent production (Bowles & Ellefson, 1985).

The samples were analysed for: (a) growth of the culture by total colony count; and (b) solvent and organic acid concentrations by gas chromatography. A Hewlett Packhard Model 5890 A chromatograph was used, and the column was packed with 6.6% Carbowax 20M 80/120, Carbopack BAW (Supelco Co. Inc.). The column was operated by increasing the temperature from 70°C to 170°C at a rate of 5°C min^{-1}, and holding at this temperature for an additional 10 minutes. The injector temperature was 200°C, the detector temperature was 300°C and the carrier gas was nitrogen with a flow-rate of 20 ml min^{-1}.

RESULTS AND DISCUSSION

In composition, labneh whey was similar to the acid whey produced by the cheese industry, namely total solids 5-6%, fat 0.3%, protein 0.9% and ash 0.7-0.8%, and only the lactose concentration (3.2 *vs* 3.8-4.2%) was lower; this difference reflects the more active fermentation of lactose by the

thermophilic bacteria in yoghurt to give a pH of 3.6 (0.9% lactic acid *vs* 0.7% in cheese whey).

Total solvent production in labneh whey at 30°C was only 4.0 $g l^{-1}$ after 7 days (Figure 1), and this low concentration could be due to the low level of carbohydrate -32 $g l^{-1}$. Alternatively, the lactose may have been metabolised only slowly, compared with glucose (Schoutens & Nieuwenhuizen, 1985), or citrate in whey may also have inhibited growth and solvent production (Maddox, 1989).

Nevertheless, it was considered more likely that the poor nutrient content of whey was leading to a low cell density and rate of metabolism and, consequently, three possible routes for improvement were considered: an increase in the concentration of lactose, hydrolysis of the lactose prior to inoculation and partial hydrolysis of the whey protein to increase the level of amino-nitrogen.

As can be seen from Figure 2, increasing the level of lactose to 64 g l^{-1} lead to a major improvement in both the total concentration of butanol in the medium and the butanol/acetone ratio. Above 64 g l^{-1}, total solventogenesis declined, and it is probable that this pattern is a reflection of cell growth. Thus, in the medium with 6.4% lactose, the lag phase was accompanied by the generation of organic acids, mainly acetic and butyric acids, to give maximum levels after 48 hours. A progressive increase in total colony count followed over the next 3 days, along with the concurrent metabolism of the acids into acetone and butanol, respectively. However, in the presence of 9.5% lactose, the cell count declined rapidly after 4 days, as did the conversion rate of acids to solvents.

Hydrolysis of the lactose did not produce any improvement in the levels of butanol or acetone compared with the control (see Figure 3). The cell counts in Figure 4 suggest that the main impact of the available glucose was manifest in a rapid increase in cell numbers after 48 hours, followed by an equally sudden decline, so that only a limited population of active cells was available to complete the solventogenic phase. Whether the clostridial cells were unable to utilise the residual lactose and/or organic acids due to catabolite repression (Monot *et al.*, 1982) was not investigated.

The hydrolysis of the whey protein gave an increased yield of total solvents compared with the control, as well as an increased cell count during the later stages of the fermentation. What was curious, however, was that there appeared to be little evidence of the cells transforming the acids into solvents, for the levels of total acids remained high over the entire 7-day period-final level of 5.5$g l^{-1}$.

Overall, it would appear that

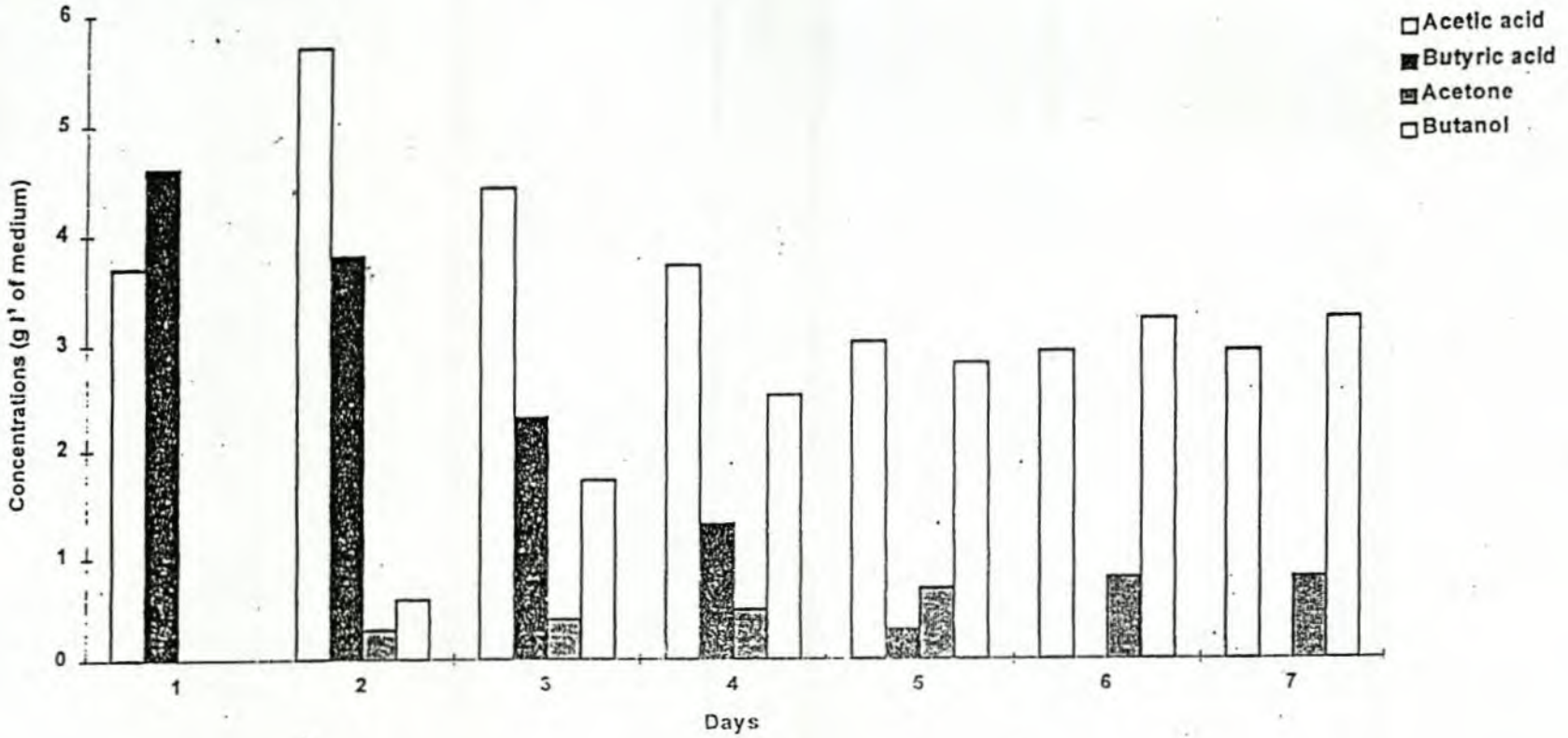

Figure 1. Concentrations of acetic and butyric acids, acetone and butanol recorded during growth of C.acetobutylicum (NCIMB 619) in labneh whey with 32 g l^{-1} lactose over a period of 7 days.

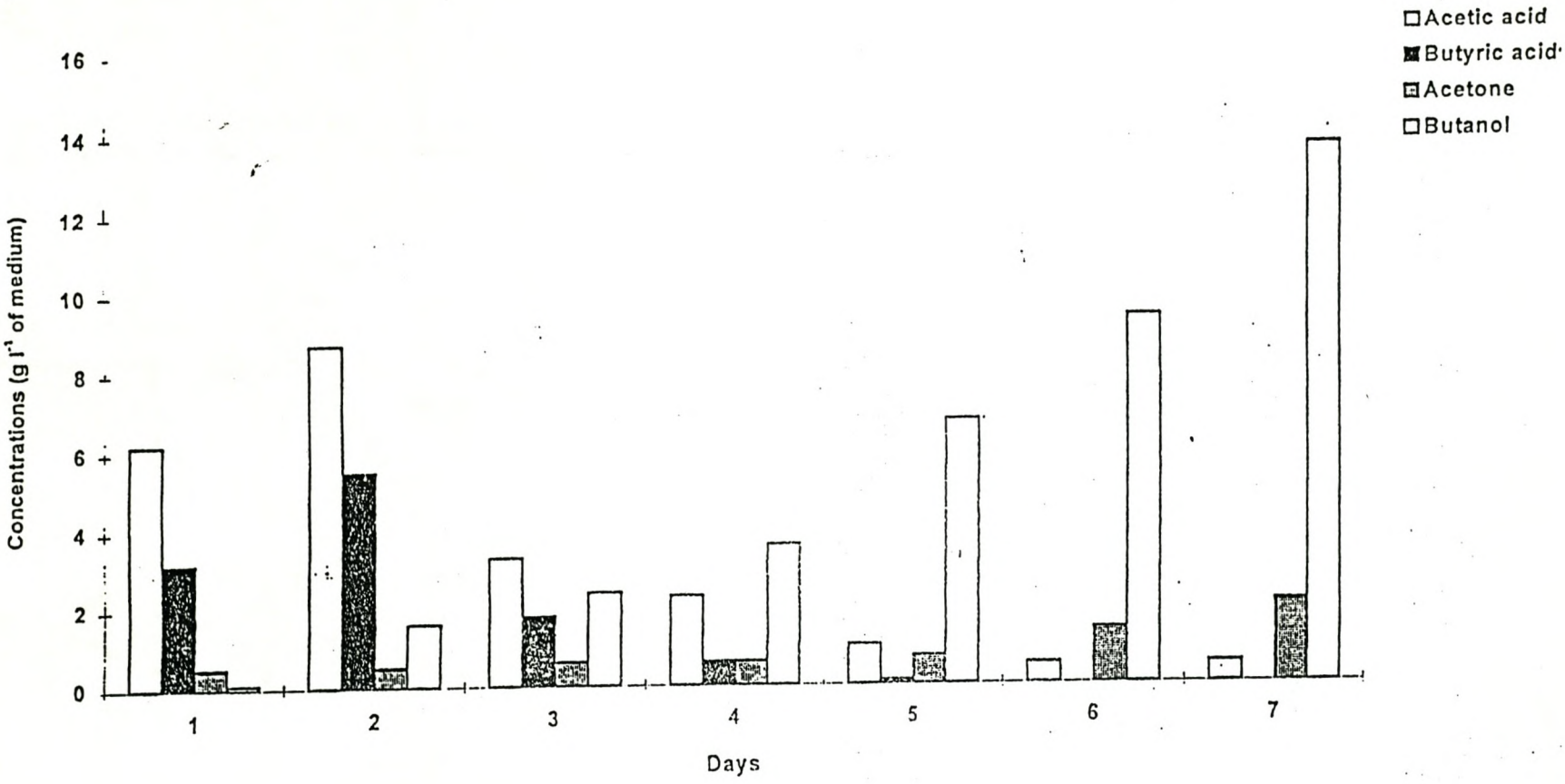

Figure 2. Concentrations of acetic and butyric acids, acetone and butanol recorded during growth of C.acetobutylicum (NCIMB 619) in labneh whey with 64 g l^{-1} lactose over a period of 7 days.

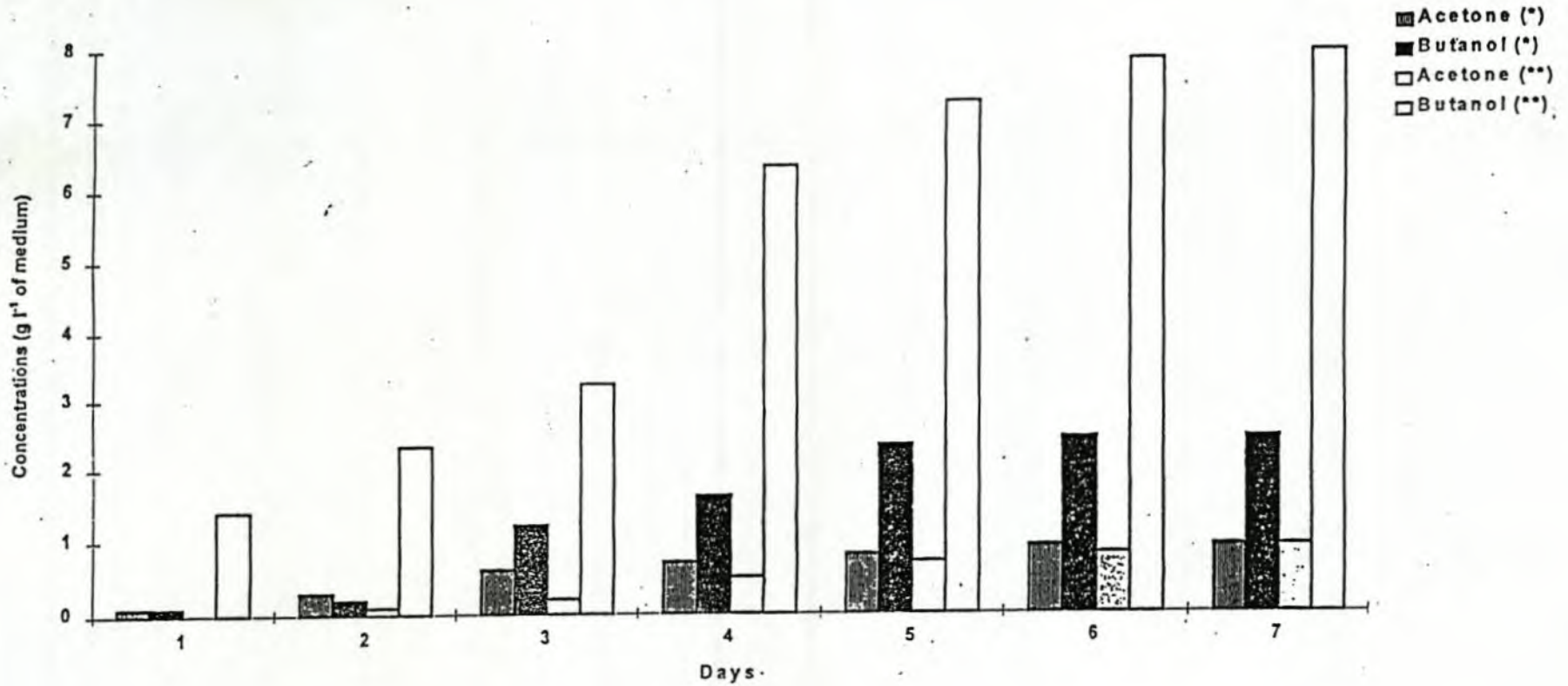

Figure 3. Concentrations of acetone and butanol recorded during the growth over a period of 7 days of C.acetobutylicum (NCIMB 619) in labneh whey (32 g l^{-1} lactose) treated with β-galactosidase (*) or pepsin (**).

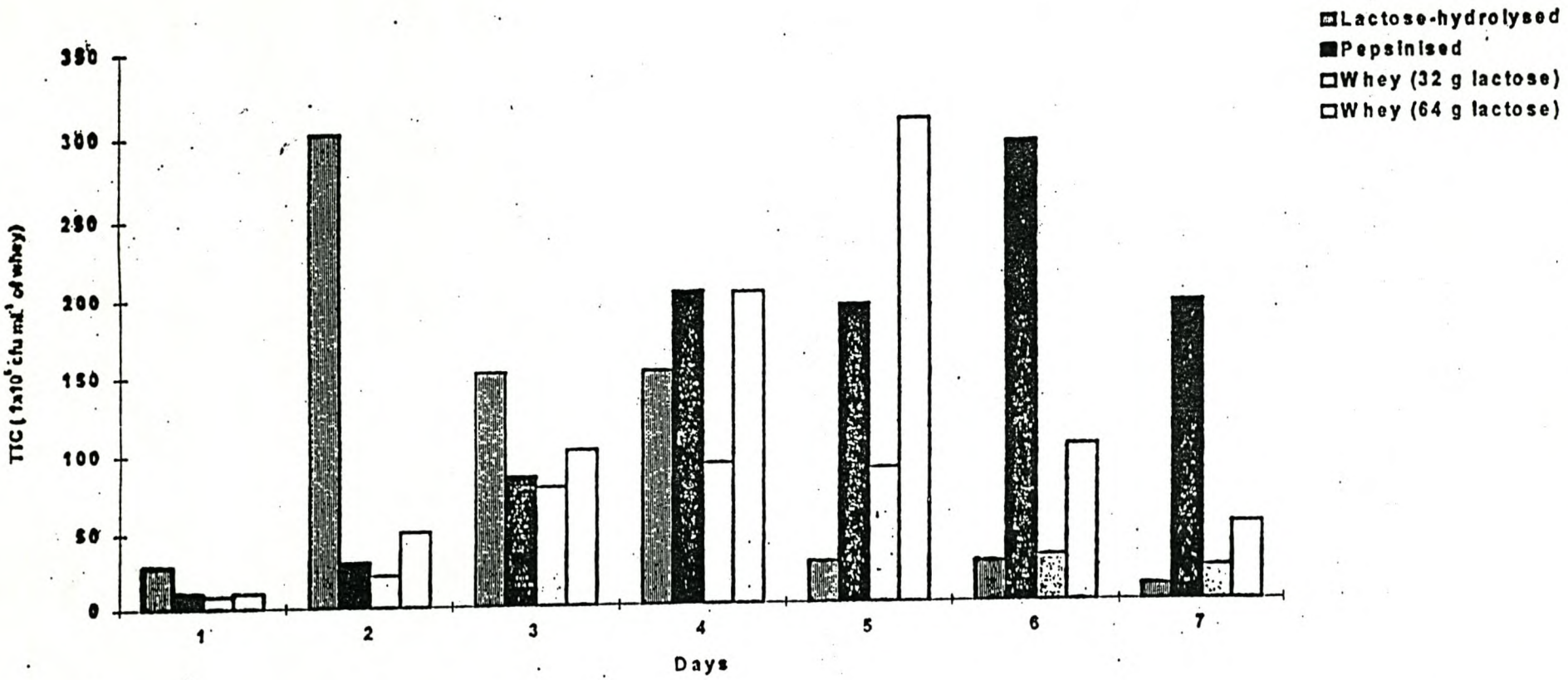

Figure 4. Mean total colony counts (TTC) recorded during the growth over a period of 7 days of C.acetobutylicum (NCIMB 619) in labneh whey with 32 and 64 g l^{-1} lactose, lactose-hydrolysed and pepsinised. Figures for TTC as colony-forming units (cfu) ml^{-1} $x10^6$ (initial counts calculated as 1 $x10^6$ cfu ml^{-1} of whey). The results are means of two trials.

labneh whey supplemented with yeast extract and lactose could support the production of butanol and acetone to a level of 15.5 $g l^{-1}$, with a ratio of nearly 7 : 1 in favour of butanol. Whether or not economics of the process would prove attractive on a commercial scale is another issue, but it is of note that a concentration of 13.6 $g l^{-1}$ of butanol could make recovery a feasible proposition. If exposure to air is sufficient to reduce the numbers of viable clostridia from the crude whey/biomass residue to an acceptable level, then this material could have a value as an animal feedstuff.

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انتاج الأسيتون والبيوتانول من تخمر شرش اللبنه

دعم الشرش الناتج من صناعة اللبنه بمستخلص الخميره (٥جم/ لتر) واللاكتوز (٣٢ جم / لتر) قبل تلقيحه بميكروب *Clostridium acetobutylicum* تحت ظروف لاهوائيه ثم التحضين على درجة ٣٠ °م لمدة سبعة أيام حيث أعطى ٦ر١٣جم/لتر من البيوتانول ، ٦ر١ جم/لتر من الأسيتون بالإضافة الى أثار من كحول الإيثيل والأحماض العضويه.

وقد اقترح أن نسبة المذيبات المتكونة تجعل استرجاعها من الوسط اقتصادى وأن هذه الطريقة ذات أهمية حيث تقلل اعتماد الدول على البترول.

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The role of olive oil in the preservation of yogurt cheese (labneh anbaris)

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Small 'balls' of yogurt cheese (40 g 100 g $^{-1}$ total solids), derived by extracting moisture from concentrated yogurt (23% total solids), can be stored in retail outlets in the Middle East (20–25°C) under virgin olive oil for up to two years. The absence of over-acidification appears to be due to the inhibitory effect of low pH (3.8), low available water (0.85) and salt (1.0 g 100 g^{-1}) on starter activity, while the anaerobic conditions created by the oil both reinforce these inhibitory factors and, additionally, prevent the growth of yeasts over the surface of the individual balls. The type of vegetable oil covering the balls was found to be unimportant, and sunflower, safflower and cottonseed oil were equally effective in preserving yogurt cheese inoculated with Kluyveromyces marxianus subsp marxianus and stored for three months at 25°C.

INTRODUCTION

Concentrated yogurt (labneh) is defined by the Lebanese Standards[1] as 'a semi-solid food derived from yogurt by draining away part of its water and water-soluble compounds'. The total solids (TS) is typically 23–25 g 100 g $^{-1}$, and the product has a cream/white colour, a soft and smooth body, good spreadability with little syneresis and a flavour that is clean and slightly acidic.[2] This perception of low acidity stems from the 'masking effect' of the high fat content of labneh—typically around 10 g 100 g $^{-1}$, for the titratable acidity may be in the region of 1.8–2.0% as lactic acid.[3] Such a level can be expected to curtail the growth of bacterial pathogens, but yeasts, moulds and some lactic acid bacteria can still cause problems of spoilage. At ambient temperatures (20–25°C), deterioration can be rapid but, even at 7°C, the characteristic signs of yeast spoilage often become visible in 7–14 days.[4] For this reason, labneh may be further concentrated to around 40 g 100 g $^{-1}$, shaped into 'balls' with diameters of 25–30 cm and placed in glass jars with a covering of olive oil. This latter product is known as labneh anbaris, and the anticipated shelf-life is in the region of two years. In rural areas of the Middle East, a similarly concentrated product, yogurt cheese, is produced and sold unpacked.[5]

The manufacturing methods and chemical compositions of concentrated yogurts have been studied by several researchers,[6–10] and processes vary from the traditional method of straining natural yogurt in a cloth bag, animal skin or earthenware vessel until the desired level of total solids has been achieved[9,11] to modern technological methods using recombination technology,[12] membrane techniques, such as ultrafiltration and reverse osmosis,[13–15] or centrifugation.[16] However, the traditional or cloth bag method remains the best known, and it is still preferred in rural areas because it does not need any costly investment.

The microbiological quality of yogurt cheese made from caprine or bovine milks (cloth bag method) and stored for 6 months under vegetable oil at room temperature was studied by Rao *et al*,[17] and they pointed out that the anaerobic conditions created by the vegetable oil, together with the low pH, caused significant reductions in the total colony and lactic acid bacterial counts, but had less impact on the yeast and mould counts. The reduction in bacterial numbers has obvious implications for product safety, and Gohil *et al*[18] concluded that the survival of *Listeria monocytogenes* in normal labneh (23 g 100 g $^{-1}$) was the result of the combined action of low pH, salt and, perhaps, an inhibitory agent(s) secreted by the starter culture. At ambient temperatures, the antibacterial effect was even more pronounced, and it was suggested that high temperature may have encouraged a more rapid diffusion of the undissociated form of lactic acid into the cells of the pathogen.

Although over-acidification as the result of prolonged starter activity could result in a loss of sensory quality of concentrated yogurt (labneh) or yogurt cheese, it would appear that yeasts and moulds are likely to be the main cause of spoilage.[4,17,19] However, there appears to be something of a conflict between the findings of Rao *et al*[17] that yeasts and moulds were largely unaffected by immersion in vegetable oil, and the shelf-life of labneh anbaris available in supermarkets. Compositional differences between the products could be important, but it is not clear whether or not the virgin olive oil used for storing the commercial brands has any special properties with

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respect to suppressing the growth of the constituent microflora of the product.

Consequently, the aim of this project was to (a) produce a yogurt cheese with 40 g 100 g^{-1} TS; (b) fabricate 'balls' of this cheese and contaminate them with a selected yeast; (c) store the contaminated balls under a number of vegetable oils; and (d) examine the growth and/or survival of the yeast over a three month period and, at the same time, monitor the survival of the starter bacteria.

MATERIALS AND METHODS

Manufacture of the yogurt cheese

A standard, freeze dried, starter culture for yogurt (Coded CH-1) containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* in a 1:1 ratio (chain:chain basis) was obtained from Chr Hansen's Laboratory Ltd, Reading, UK. A working culture was prepared by inoculating a batch of sterilized (121°C/2 min), reconstituted skimmed milk powder (100 ml, 12 g 100 g^{-1} TS) with 0.2 g of the freeze dried culture. After incubation at 42°C until the onset of gelation—around pH 5.0—the culture was then maintained at 5°C overnight. Two subcultures (2 ml 100 ml^{-1} sterile milk) ensured full resuscitation of the freeze dried material, and the gelled culture was then stored in the refrigerator at 4°C for one week before routine subculturing.

The basic yogurt was made from full cream, spray-dried milk powder (Adams Food Ingredients, Leek, Staffs, UK) reconstituted at 50–55°C to an initial total solids of 16 g 100 g^{-1} using a high speed emulsifier (Silverson Machines Ltd, Chesham, Bucks, UK). The milk (4 l) was then heated at 85°C for 30 min, before cooling to 45°C. When the temperature had equilibrated, the milk was inoculated at a rate of 2 ml 100 ml^{-1} with a fresh culture, and the mixture was stirred for 5 min prior to incubation at 42°C to pH 4.3.

After cooling the natural yogurt under refrigeration, it was mixed manually for 5 min and 3 kg were placed into a cloth bag prepared from a double layer of cheese cloth. The bag was then hung in a cold room at 4°C for 16 h until the TS of the strained yogurt reached 23–24 g 100 g^{-1} as calculated from the volume of whey removed. The final manipulation to produce yogurt cheese involved transferring the strained yogurt into a cloth bag (four layers of cheese cloth) placed in a perforated cheese mould (18 cm diameter). Concentration of the product up to 40 g 100 g^{-1} TS level was achieved using a screw-press with gradually increased pressure over a period of 6 hours at room temperature.

Fine, dry salt was blended into the cheese at a rate of 1 g 100 g^{-1}, and 'lumps' (approximately 20 g) of the product were rolled by hand into the traditional balls employed in labneh anbaris (see Fig. 1).

Fig. 1. A jar of 'balls' of yogurt cheese stored under virgin olive oil.

Inoculation of the yogurt cheese

Initially, *Kluyveromyces marxianus* subsp *marxianus* was grown on slopes of Potato Dextrose Agar[20] but, prior to inoculation of the yogurt cheese, tubes of Malt Extract Broth (10 ml) were inoculated and incubated for 48 hours at 30°C. The cell count in the broth was enumerated on acidified Malt Extract Agar to obtain an estimated number of each organism per ml of suspension, and it was calculated that, if each ball was inoculated with 20 µl from the first dilution in sterile peptone water (0.1%), a concentration equivalent to 7.5×10^4 cells of *K marxianus* subsp *marxianus* g^{-1} of cheese would be obtained. Consequently, batches of balls were inoculated by inserting the tip of a syringe into the centre of each ball along a channel made with a sterile needle, and depositing 20 µl of the suspension; removal of the needle allowed the exit point to close naturally.

To test whether virgin olive oil (Filippo Berio, Lucca, Italy) had any detectable antimicrobial properties, three commercial vegetable oils—namely, sunflower, safflower and cottonseed (Anglia Oils Ltd, Hull, UK)—were chosen as control oils; the latter oils are all refined, and hence free from the phenolic and other compounds that could possess mycostatic/mycocidal properties. Glass jars with rubber seals (0.75 l) were sterilized in sodium hypochlorite solution (3 mg ml^{-1} of available chlorine) for 30 min, rinsed with sterile distilled water and oven dried prior to use. Three jars were then filled with 'balls' of cheese (20/jar), and the jars filled with virgin olive oil so as to cover the balls to a depth

of around 3 cm. The same procedure was repeated with the sunflower, safflower and cottonseed oils, and three additional batches without any covering of oil provided an additional control.

For enumeration of the starter bacteria, a further three jars were filled with 20 balls of cheese uninoculated with yeast, and a covering of virgin olive oil was added. All the jars were stored at 25°C.

Analysis of the samples

At 15, 30, 45, 60, 75 and 90 days, two balls of yogurt cheese were removed aseptically from each of the three jars containing the different oils, as well as from the three jars without oil, for enumeration of *K marxianus* subsp *marxianus*. On each occasion, the two balls of cheese (approximately 40 g) were drained of excess oil on filter paper, and then transferred to 360 ml of 0.1% peptone (at 45°C) in a stomacher bag.[21] After blending for 1 min, the mixture was taken as the first dilution, and the yeast counts determined according to the method described by the International Dairy Federation (IDF).[22]

The uninoculated samples were tested at the same time but, in this case, duplicate 0.1 ml aliquots from dilutions between 10^{-4} and 10^{-6} were spread onto plates of Tryptose Proteose Peptone Yeast Agar with Prussian Blue (TPPY-PB) for enumeration of *Strep thermophilus* and *Lac delbrueckii* subsp *bulgaricus*.[23] The entire trial was repeated three times but, because the decline of the starter culture was so rapid, sampling of the batches under olive oil (without yeast) was completed at 3, 6, 9, 12 and 15 days during the second and third trials.

Chemical analyses of the original yogurt, concentrated yogurt and yogurt cheese included pH using a model Kent EIL 7045/46 meter fitted with a standard combination glass electrode, titratable acidity as determined by the IDF,[24] TS content according to IDF,[25] fat content by the Gerber method using Gerber (scale 0–8%) butyrometer tubes as cited in the British Standards Institution method[26] and available water as measured using the Dew Point Meter (DP 383R, UK) at 25°C the relative humidity was multiplied by 100 and expressed as a_w. Protein and lactose were by infrared absorption.[27] Similar procedures were applied to the whey expelled during processing.

Statistical analysis

The results were analysed in Exel (Windows 95) to obtain mean values and standard errors, and the inhibitory activity of the oils against yeasts was tested using the *t* test—Assuming Unequal Variances.[28]

RESULTS AND DISCUSSION

Properties of the yogurt cheese

The mean results for TS, fat and titratable acidity from two separate trials are presented in Table 1, and the trend in concentration follows the expected pattern; the a_w of the yogurt cheese was measured as 0.85.

The chemical composition of the test yogurt cheese was also compared with one commercial sample obtained from a market in Qatar which contained (100 g^{-1}) 22.0 g fat, 20.0 g protein and 7.60 g lactose, compared with the laboratory made product, which contained (100 g^{-1}) 18.25 g fat, 15.0 g protein and 5.50 g lactose; the pH measurements for the two materials were 3.95 and 3.80, respectively. The differences between the samples reflect both the differences between the TS of the two products (nearly 50% TS in the sample from Qatar) and, probably, contrasted raw materials and/or methods of production. Nevertheless, the relationship between the main constituents suggested that the test material was broadly comparable with products on the market, and hence would provide a suitable material for the evaluation of microbial spoilage.

TABLE 1

Some characteristics of the reconstituted milk, the yogurt, the concentrated yogurt and the yogurt cheese, along with those of the whey expelled during drainage of the yogurt and the pressing of the concentrated yogurt; all figures as g 100 g^{-1} and acidity calculated as lactic acid (%)

Samples	*Total solids*	*Fat*	*Titratable acidity*
Milk	15.84 ± 0.02	4.45 ± 0.15	ND
Yogurt	15.02 ± 0.26	3.94 ± 0.44	1.06
Concentrated yogurt	24.20 ± 0.12	8.53 ± 0.02	ND
Yogurt cheese	40.45 ± 0.15	18.25 ± 0.25	1.23
Whey (drainage)	8.32 ± 0.01	0.50	ND
Whey (press)	7.94 ± 0.13	0.40	1.54

ND = not determined.

Survival of *K marxianus* subsp *marxianus*

The results shown in Fig. 2 suggest that the cell counts of *K marxianus* subsp *marxianus* increased over the first 15 days of storage from the initial estimated count of 7.5×10^4 colony forming units g^{-1}, but then declined over the next 45 days. At that point, some marginal differences in the behaviour of the survivors began to emerge. Thus, while the counts under olive oil remained stable, the colony counts showed a slow but continual increase under cottonseed oil. Under sunflower oil, the inhibitory action of the oil appeared to be lost after 75 days.

However, the differences at 90 days were not statistically significant, so that it would appear that the phenolic and other compounds in virgin olive oil are not contributing to the gradual loss of viability of the yeast. This view is supported, to some extent, by the decline in cell numbers in the yogurt cheese stored without any covering of oil, for it would appear that the reducing counts are the result of a cumulative impact of factors associated with

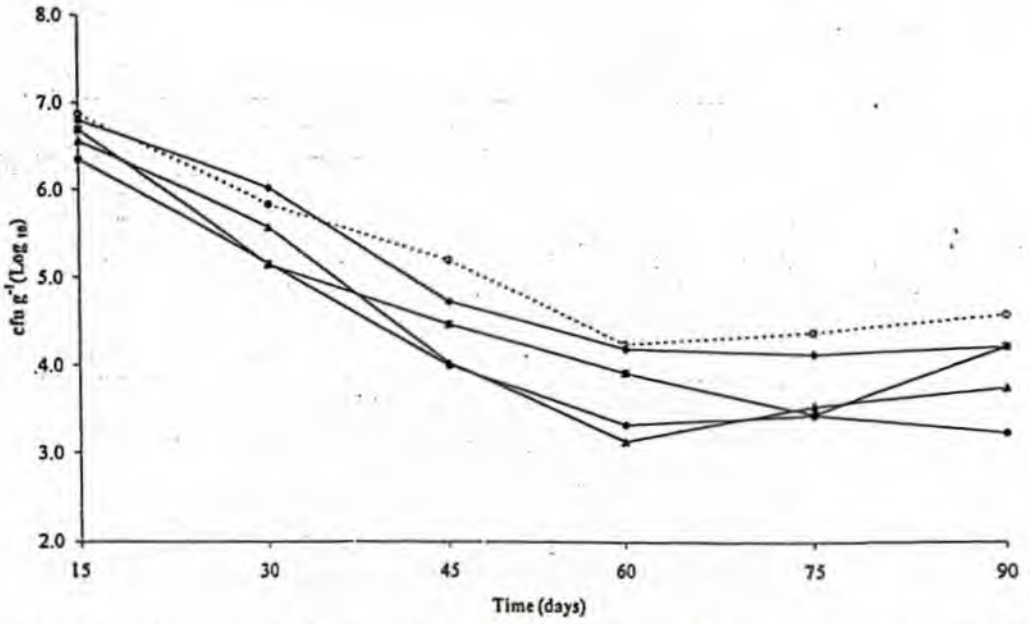

Fig. 2. The growth/survival of *K marxianus* subsp *marxianus* during the storage of yogurt cheese under vegetable oils at 25°C. ◆ Olive oil, ■ sunflower oil, ▲ cottonseed oil, ● safflower oil, O without oil. All values are means of three trials.

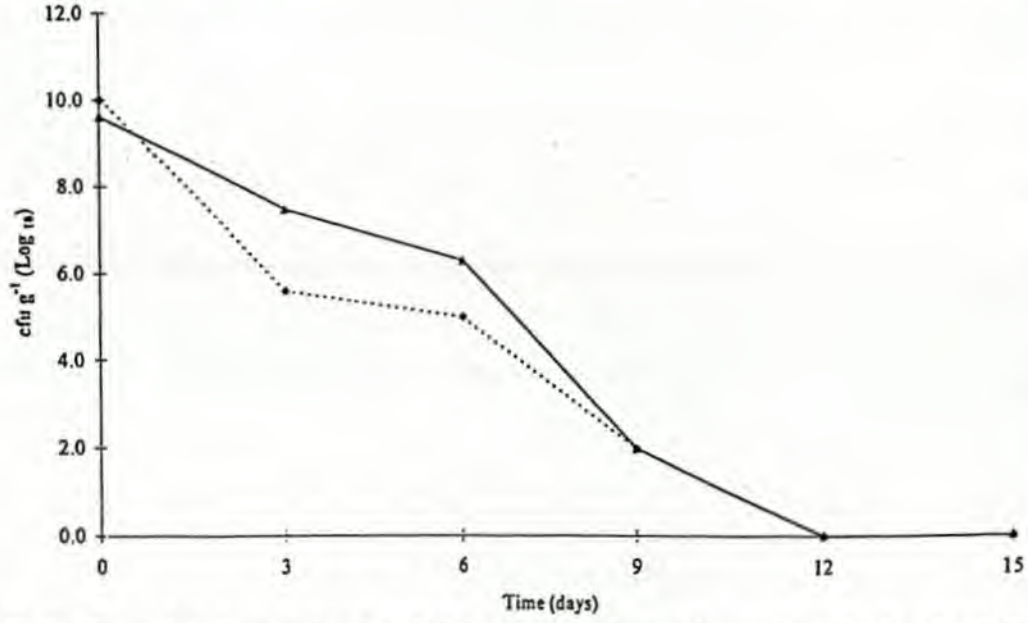

Fig. 3. The survival of starter culture bacteria during the storage of yogurt cheese under virgin olive oil at 25°C. ◆ *Strep thermophilus*, ▲ *Lac delbrueckii* subsp *bulgaricus*. All values are means of three trials.

the cheese per se, ie, low a_w, low pH and, possibly, the presence of antimicrobial compounds secreted by the starter culture. However, it may be relevant that the initial location of the yeast cells was in the centre of each ball, and the counts might well have increased dramatically had some of the cells migrated into a zone of less severe inhibition, eg, surface water and adequate aeration.

Enumeration of starter culture

This latter point is supported, albeit indirectly, by the survival of *Strep thermophilus* and *Lac delbrueckii* subsp *bulgaricus* in yogurt cheese under olive oil for, as shown in Fig. 3, the conditions within the balls caused a rapid decline in bacterial counts. Clearly the anaerobic conditions imposed by the oils and, perhaps the presence of phenolics and fatty acids in the vegetable oils, could be contributory factors, but the rates of inactivation suggest that the role of the latter factors is minor.

Conclusion

Overall, this study has confirmed that the immersion of balls of yogurt cheese under

vegetable oil is important with respect to the control of any superficial growth of yeasts and moulds. However, the retention of the organoleptic quality of the cheese appears to be due to the nature of the product, for the total loss of viability of the starter culture means that over-acidification is unlikely to be a problem. Similarly, the decline in numbers of yeasts suggests that the development of off-flavours should be minimal, and hence it is not surprising that labneh anbaris can be sold with a projected shelf-life of two years. It would appear also that the use of olive oil is simply a matter of local availability, and that other vegetable oils could provide equally adequate protection against the growth of surface contaminants.

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THE POTENTIAL VALUE OF YOGHURT-CEREAL MIXTURES

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The production of sun-dried, yoghurt-cereal mixtures in the Middle East enables the limited supplies of milk to be conserved in rural areas. The protein-rich material also forms a valuable dietary ingredient during periods of the year when fresh milk is unavailable.

More extensive use of these products is limited by their rather acrid flavour, caused primarily by the uncontrolled nature of the fermentation. This study showed that when pre-cooking of the wheat was allowed to cause extensive destruction of the microflora, then the bacteria in the yoghurt, namely *Streptococcus thermophilus* and *Lactobacillus bulgaricus* became responsible for the subsequent acid development. Precooking can therefore control the final acidity of the product and, to some extent, its flavour, and fermentation can be stopped at any pre-selected point. The complete cessation of microbial activity was achieved by roller-drying the finished mixture. This process limited the losses of B-vitamins and imparted a pleasant, malty flavour to the final product.

On reconstitution with hot water it was acceptable to a wide range of palates. Production of yoghurt-cereal mixtures should be encouraged in those areas of the world where milk is an under-utilised commodity, particularly as the process could be readily standardised for an "intermediate technology" situation.

KEY WORDS: Yoghurt, cereals, fermented foods, kishk, milk, wheat.

Most industrialised countries have well established national policies with regard to their dairy industries. In countries like Denmark (Petersen, 1963), United Kingdom, Netherlands and Germany (Anon, 1960) the agricultural economy has, for many years, been oriented largely towards milk production, and the emergence of co-operatives involving both farmers and companies means that nearly 79 percent of all milk is delivered in a fresh condition directly to processing plants. The situation in developing countries is very different and Table I illustrates the disparity between the contrasted areas.

The utilisation of milk by the different countries varies widely depending on demand patterns, processing facilities, marketing systems, retail prices and income of the consumer, and average consumption per caput varies from only a few kilograms per annum in some developing countries to a maximum of 290 kg/person/year in Finland (Food and Agriculture Organization, 1969).

It is clearly in developing countries that the real need for efficient milk production and utilisation still exists, and with some 80 percent of the population living in rural areas, industrial solutions are almost irrelevant. It is, therefore, of primary importance to promote the economic and social development of these rural populations, but to do so in a manner that affords the minimal amount of disruption to the traditional patterns of life. Thus, in some areas, herds of cattle consisting of hundreds of animals are considered merely as a

TABLE I

Recorded milk production (1000 metric tonnes) in industrialised as compared with developing countries

Area	1960–65 (average)	1973	1974	1975
Industrialised[a]	190,818	201,195	201,853	202,003
Developing[b]	38,851	48,218	50,207	52,182

[a]Including North America, Europe, Australia, etc.
[b]Africa, Latin America, Near and Far East. (After: FAO, 1975.)

sign of wealth, and are used neither for meat nor milk production. In such a situation the potential for change is evident, as the losses to the community of valuable milk protein must be very high indeed. If, in addition, it is accepted that at least 30 percent of the fresh milk that is collected goes to waste, then the case for initiating improvements is very strong indeed.

TRADITIONAL PRACTICES EMPLOYED TO PREVENT SPOILAGE

In warm climates, the production of milk tends to be seasonal, and with temperatures as high as 40°C, the milk turns sour and coagulates within a short time of milking. If this process happens by chance, then the end-product is frequently rather unpleasant to eat or drink. However, if the milk is boiled immediately after collection, and mixed with a portion of sour milk from the previous day, the new sour milk will have a distinctive character and flavour, and the coagulum will be thick and uniform. Products of this type are collectively referred to as yoghurt, although many traditional names are still in common use.

Although yoghurt is an attractive product for the consumer, as well as being hygienically safer than raw milk, its keeping quality at ambient temperatures is limited. To overcome this problem, some of the yoghurt produced during the months of the year when milk is available is turned into condensed yoghurt, and Figure 1 illustrates a few of the available processes and products.

The derivations of these additional foods is obviously an advantage, but the basic concentrated yoghurt is still prone to spoilage through excess acid development or microbial contamination, while those materials preserved under oil may not be acceptable to all households. The most attractive process may be, therefore, that in which the concentrated yoghurt is dried in conjunction with a cereal flour, and the advantages of this idea may be summarised as:

the dry products are virtually non-hygroscopic, and hence can be stored at ambient temperature with few problems;

when ground and reconstituted with hot water, the resultant porridge is widely accepted, especially by young people (van Veen and Steinkraus, 1970);

the nutritional value of the porridge makes it a valuable supplement to any carbohydrate-rich diet (Frankul and Pellett, 1959);

the process provides a simple means of prolonging the availability of milk protein in those areas with seasonal production.

There are, therefore, good reasons for proposing that the use of yoghurt-cereal mixtures should be encouraged, and it is the aim of this paper to consider what is currently known of these products, and to suggest ways in which their usage might be extended.

YOGHURT-CEREAL MIXTURES

One of the best known of these materials, Kishk, is already widely consumed in the rural areas of Iraq (Frankul, 1961), while a similar product, Tarhana is to be found in Turkey (Koksal, 1961).

The basic process starts with the treatment of the cereal, normally wheat, and, after washing to remove any foreign materials, the grain is boiled for an hour or so in open kettles. This process gelatinizes the starch (Neufeld, Weinstein, and Mecham, 1957), so altering its solubility through the formation of amylose and amylopectin complexes (Priestly, 1976a, b, 1977). The parboiled grain is then spread on the ground to dry, and, being a mid-summer activity, the sun rapidly lowers the moisture content to around 12 percent. After removing the chaff, the grain is then milled into a coarse flour ready for storage, and it is probable that a period of four to six months can be achieved before rancidity becomes a problem.

The preparation of this parboiled wheat, or *bulgur* as it is known locally, is one aspect of the process that would appear to merit closer control. Thus, Saracoglu (1953) found that bulgur produced in Turkey had an average thiamine content of 27 percent less than the original wheat. Adolph, Shammas, and Halaby (1955) reported similar losses for niacin; riboflavin was reduced by up to 75 percent. It is interesting, however, that the growth of rats fed on whole wheat as compared with bulgur was the same (Shammas and Adolph, 1954), and hence the losses revealed by chemical analysis may not be significant in practice. It is also evident that raw flour could not be employed for other reasons, because not only would the reconstitution of the ungelatinised starch prove difficult, but the flavour of uncooked meal could also prove unattractive.

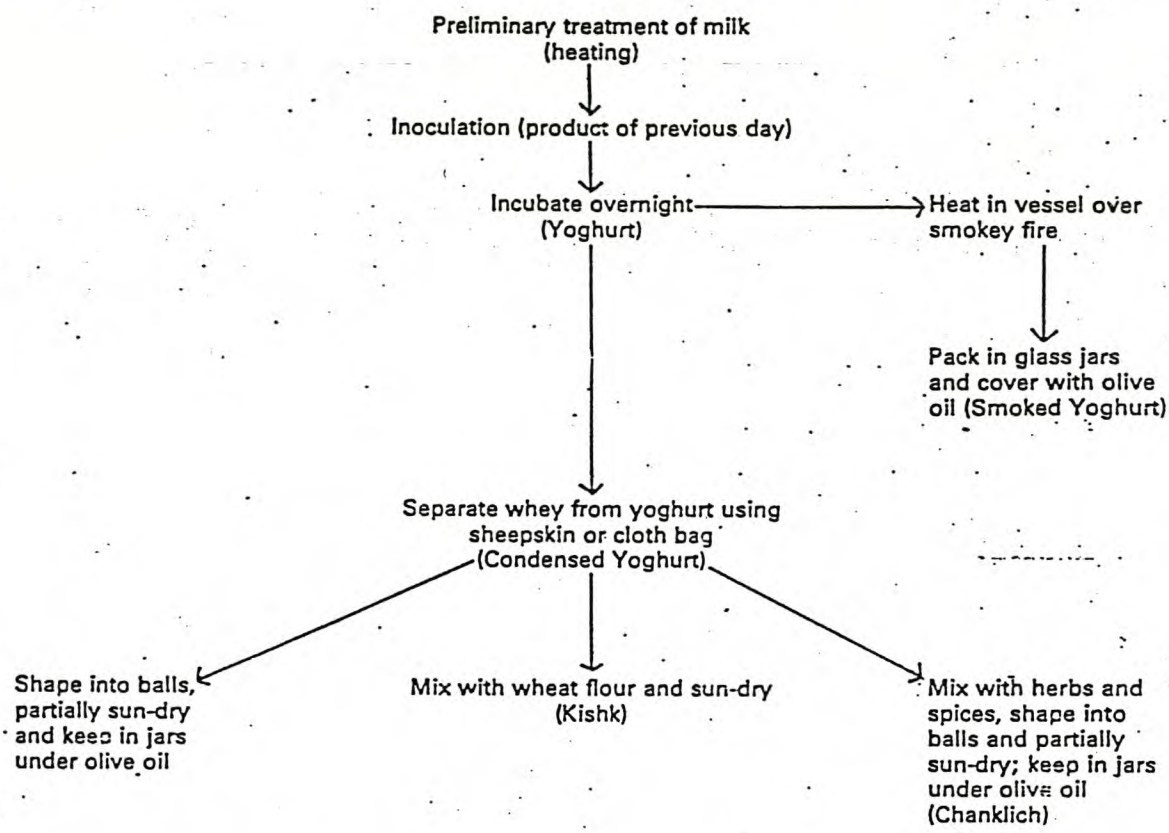

FIGURE 1 Traditional production of yoghurt and some related products.

The final stages of producing kishk involve mixing the ground parboiled wheat into the concentrated yoghurt (see Figure 1), and then allowing the dough-like mix to ferment for up to five days. What microbiological processes are involved in this stage are still largely unknown, but, traditionally, the fermentation is simply allowed to run its natural course, and the finished dough is then turned into small rolls, about 70–80 cm in diameter, and sun dried.

In an alternative process, two/four parts of normal yoghurt are mixed with one part of cereal flour, and the mixture is then allowed to ferment for 5–7 days at ambient temperature (30–40°C). At this time, an additional quantity of fresh yoghurt is added to the mix, and the fermentation is then allowed to continue for a further 4–5 days. At this point, the wet kishk is spread out on cloths to dry in the sun for 1–2 days.

The exact nature of the process is probably a matter of tradition, but, whatever the procedure, these products form an extremely important component of the diet for certain communities. To many palates, however, the acrid nature of the reconstituted material would be quite unacceptable, whereas if a similar product could be devised having more widely attractive organoleptic characteristics, then the process could well be of interest to other developing countries where the handling of raw milk presents a problem. It was decided, therefore, that an attempt should be made to develop a yoghurt-cereal product with the essential advantages of kishk, but without the concomitant drawbacks over acceptability.

LABORATORY PRODUCTION OF A KISHK-LIKE FOODSTUFF

The basic process is shown in Figure 2, and it differs from the traditional method mainly in the second fermentation stage. Thus, although the

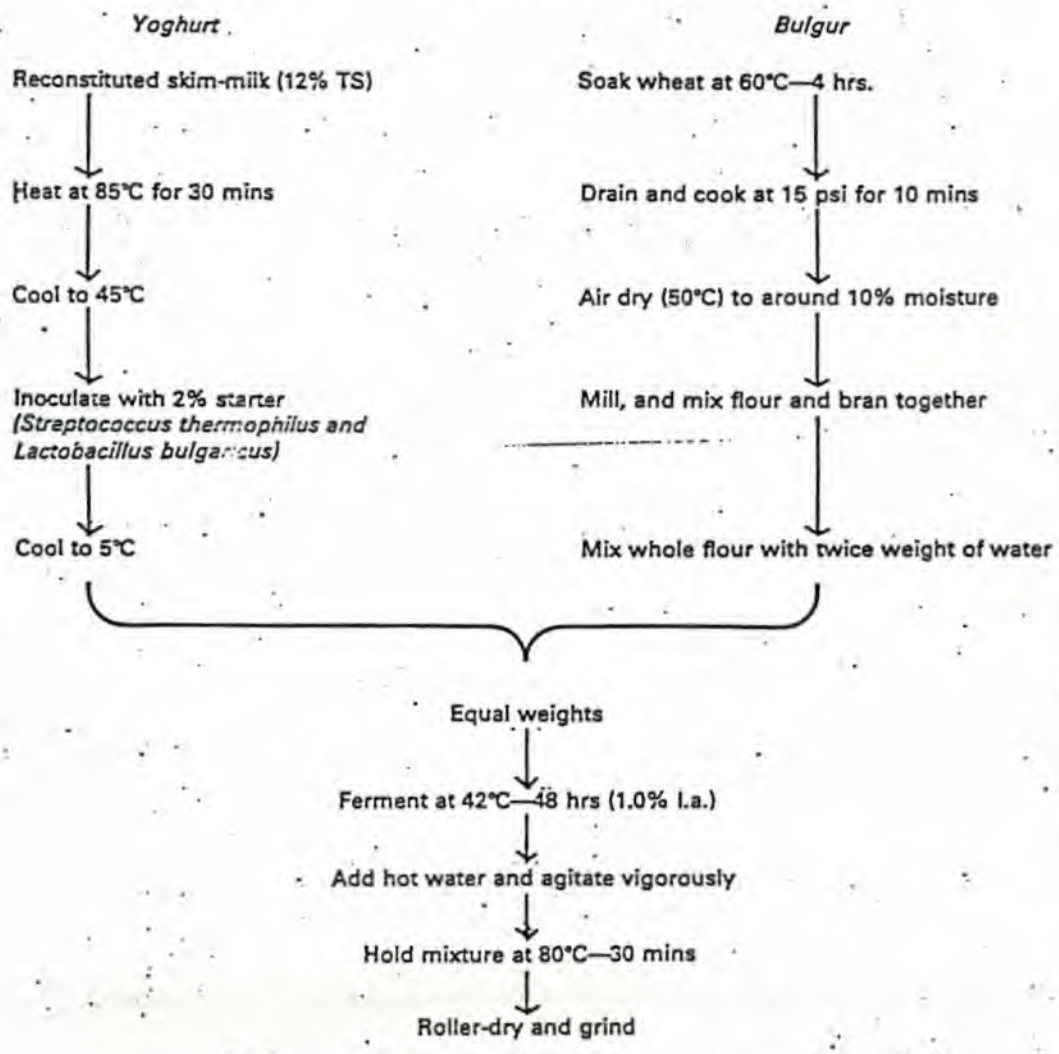

FIGURE 2 Flow diagram of the method of producing a "kishk-like" product on a laboratory-scale.

yoghurt base was made with an acidity of around 1.5 percent lactic acid, after mixing with the flour and re-incubating, the acidity of the final product was held at 1.0 percent l.a. This mild acid development during the second fermentation appears to be a consequence of restricting, by heat-treatment, the lactic microflora from the wheat flour. The result of this reduced acidity, (1 percent as compared with 2.5 percent lactic acid for its native counterpart), is that the end product has, when mixed to a porridge-like consistency with hot water, has a mild, agreeable flavour that suits most tastes.

The employment of roller-drying, or even spray-drying, for the final preparation appears to offer some advantages, over sun-drying, by maintaining the riboflavin content of the product, and it was also noted that the porridge acquired a slightly malted flavour in the process; an improvement that was enhanced by the addition of 20 percent malted wheat flour to the basic ingredients. The actual malting process was similar to that described by Fleming, Johnson, and Miller (1960), and the effect of this process on the nutritional value of kishk is shown in Table II.

The increased level of riboflavin is not unex-

TABLE II

Some typical analyses of laboratory prepared yoghurt-cereal mixtures, and some comparative data from the sources cited

Protein (%)	Ash (%)	Niacin	Riboflavin (all mg/100g of product)	Thiamin	
18.2	2.3	3.2	0.3	0.1	This study[a]
18.3	2.2	3.5	0.6	0.2	This study[b]
29.4	11.6	2.9	0.4	0.06	This study[c]
18.4	—	3.7	0.2	0.2	Frankul (1961)[d]
17.4	—	3.5	0.05	0.2	Frankul (1961)[e]
14.8	—	—	0.3	—	van Veen, Graham, and Steinkraus (1968)
23.5	8.1	3.5	0.27	—	El-Sadek *et al.* (1958)
23.5	8.1	—	—	—	Morcos *et al.* (1973)

[a]Yoghurt + normal cooked flour.
[b]Yoghurt + malted flour.
[c]Village sample from Iraq.
[d]Laboratory sample.
[e]Village samples from Iraq.

pected (Stringer, 1946), but it is noticeable that the other B-vitamins also showed an improved presence. The attractive malty flavour was obviously more distinct, but it was also observed that acid development during the kishk fermentation was more rapid. Thus, a titratable acidity of one percent lactic acid was reached in approximately 24 hours, whereas, with the normal cooked flour, the same acidity was reached one day later. This difference may well be a reflection of the production of free sugars during the malting process, for while 250 mg/100 g of flour was found in normal flour, this figure reached 831 mg/100 g in the malted flour. At least some of these sugars would have a stimulating effect on the dominant lactic microflora (namely, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*), and it is clear, therefore, that the secondary fermentation stage in the production of kishk could be rationalised considerably.

It will be important to determine, of course, whether the lower acidity will adversely effect the keeping quality of the product at high ambient temperatures viz-a-viz the materials of village origin. Nevertheless, it is fair to conclude that the kishk-type foodstuff described above could well have a much wider consumer appeal than its native counterpart, while, at the same time, retaining its essential nutritive value. If this view is correct, then it is to be hoped that an approach of this nature, to the problems of milk preservation and distribution, will make it possible for those areas of the world that lack modern technology to utilise their sparse supplies of milk protein to better advantage.

CONCLUSION

The widespread acceptance of yoghurt-cereal mixtures in certain areas of the Middle East, and their obvious value as a means of preserving valuable milk protein from spoilage, suggests that an expansion of their use in developing countries would have much to recommend it. Whether this expansion involves the introduction of the material into new rural areas, or the rationalisation of production in communities becoming urbanised is of little consequence. What is important is that the process offers a means of conserving the limited resources of available raw milk, and, in addition, provides the community with an attractive and nutritionally valuable dietary ingredient.

It must be admitted, of course, that some of the native products are of limited organoleptic appeal outside their country of origin, but this problem is really a matter of process control. Once the necessary refinement has been achieved, then there seems no reason to suppose that yoghurt-cereal mixtures could not prove to have a more ubiquitous appeal.

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THE ACCEPTABILITY OF YOGHURT-CEREAL MIXTURES TO A RURAL COMMUNITY IN MEXICO

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A dried yoghurt-cereal mixture was produced with a final protein content of around 17 per cent, and an amino-acid spectrum close to the FAO/WHO standard: lysine and threonine were the limiting values. Feeding trials with rats confirmed this slight deficiency, in that the mixture had a PER value of 2.3 (casein—2.5) and an NPU value of 61.2 compared with 62 for casein.

The acceptability of the product was tested by substituting the mixture for rice flour in a typical Mexican food, *Atole*. This gruel is widely consumed in rural communities, particularly by children, and the yoghurt-based equivalent was readily drunk by mothers and children alike. Flavoured gruels, especially strawberry and vanilla, proved most popular. It was concluded that the production of yoghurt-cereal flours could be a valuable method of preventing losses of liquid milk in rural areas, and that the material could form a useful dietary adjunct, especially for pre-school children.

KEY WORDS: Yoghurt; cereals; fermented foods; milk; Mexico; food processing.

INTRODUCTION

There are many reasons put forward to explain why malnutrition is an ever-present problem in certain areas of the world, and yet in spite of considerable activity by the United Nations and other bodies, solutions are not readily forthcoming. One of the most depressing aspects of this situation is that children are often the most vulnerable group in terms of dietary deprivation. Thus, in Mexico for example, some 32.3 percent of all children show, at around one year, some degree of malnutrition, and a survey of six-year-olds put the percentage malnourished at around 50 (Chavez, 1974). The effect of this malnutrition will vary with the individual, but it is probable that many victims will display the classic symptoms of retarded growth, diminished resistance to infection and impairment of the mental processes, (Martinez and Chavez, 1967).

If this conclusion is correct, then remedial action becomes a priority, but, at the same time, any proposed solution should be sustainable within the economy of the area. It is in this context, therefore, that the employment of yoghurt-cereal mixtures could be relevant (Robinson and Cadena, 1978), because up to 30 percent of milk produced in the rural areas of Mexico is currently wasted (Anon, 1970). If this waste could be eliminated, and the valuable protein made available to pre-school and older children, then it is possible that the worst ravages of malnutrition could be diminished, and at a modest cost.

A method for the production of yoghurt-cereal mixtures has been discussed elsewhere (Robinson and Cadena, 1978), and it is the aim of the present paper to show first that the observed nutritional value of one such material confirms that it would be a useful addition to cereal-based diets; and second that foods incorporating yoghurt-cereal mixtures could prove popular in rural communities.

MATERIALS AND METHODS

Although it is envisaged that whole milk would be employed for normal production, a commercial sample of dried skim milk (33.7 percent crude protein) was used to make the basic yoghurt. Wheat (11.2 percent crude protein) was used for the preparation of the "bulgur," and, after the second incubation, the finished mixture was made into a "slurry" and vacuum-dried. Some 50 kg of yoghurt-cereal mixture were produced in this manner, and then finely ground to facilitate incorporation into any suitable item of the traditional Mexican diet.

The expected vitamin contents had been established previously, so that chemical analysis of the

mixture was confined to protein, ash, fat, crude fibre and moisture. The general methods suggested by the AOAC (1970) were employed for this study, but, because a portion of the protein is derived from wheat, the value for the crude protein content of the mixture is subject to some inaccuracy. However, previous investigators (Frankul, 1961; van Veen, Graham and Steinkras, 1968) have also employed the formula ($N \times 6.25$) to estimate the protein content of yoghurt-cereal mixtures, and hence, for comparative purposes, this convention has been continued.

The biological value of the product was found by measuring the net protein utilisation (NPU) in rats (Miller, 1963), and the protein efficiency ratio (PER) employing the method of Campbell (1963). The experimental diet was formulated so that the animals received ten percent protein in the form of the yoghurt-cereal mixture; casein was used in the control diets. Male rats (Wistar strain) were used as the test animals and each animal weighed 35–40 g at the start of the experiment. Each animal was individually caged for the duration of the test (28 days), and ten rats were fed on a diet including the yoghurt mixture, and ten on control meals. The weight of food consumed was recorded for the duration of the experiment, and each rat was weighed three times per week to determine the increase in body weight. The data was then analysed according to the methods outlined by Snedecor and Cochran (1967), and the results expressed as mean values ± standard error of the mean (SEM).

The amino-acid spectrum of the material was also examined, for although chemical analysis *per se* is not always a valid indicator of quality, it can indicate the cause of any observed deficiency. Consequently, six samples of the yoghurt mixture were weighed out, so that each one contained roughly 12 mg of nitrogen. Duplicates were then analysed for: total nitrogen (AOAC, 1970); basic and acidic amino-acids using a technique based on the method of Spackman, Stein and Moore (1958); tryptophan was estimated employing the technique of Spies and Chambers (1949); sulphur amino-acids involving a pre-oxidation procedure with performic acid.

This procedure gave a clear indication of the amino-acid spectrum of the product, so that, although confirmatory analyses of subsequent batches may be desirable, it was decided to limit attention in the first instance to that material required for the biological assays. One of the essential aims of the project was, of course, to monitor the sensory reaction of children to the mixture, and hence a precautionary microbiological analysis was carried out employing methods derived from those of the American Public Health Association (APHA, 1972); in particular an assessment was made for total viable count and coliforms.

RESULTS OF LABORATORY ANALYSIS

The results of the chemical analysis of the yoghurt-cereal mixture are shown in Table I, along with the analyses of the starting materials. It is clear from the Table that, if the yoghurt-cereal mixture were to replace a straight cereal flour, then both the mineral and protein components of the food would be improved. The quality of the protein was also enhanced, and, as shown in Table II, only lysine and threonine could become limiting if the yoghurt mixture were to be employed as a sole source of protein.

TABLE I

Chemical analysis of a yoghurt-cereal mixture, and of the skim-milk powder and ground Bulgur used in its manufacture; (g/100 g.)

	Skim-milk	Bulgur	Yoghurt-cereal
Moisture	3.5	11.2	6.0
Ash	8.0	1.4	2.5
Protein	33.7	11.1	17.1
Fat	1.5	2.0	1.7
Crude fibre	—	2.0	2.2
Carbohydrate	53.3	72.3	70.6

This conclusion was borne out by the feeding trials with rats, and the figures for both Protein Efficiency Ratio and Net Protein Utilisation confirmed the dietary value of the yoghurt-cereal mixture. It is accepted that all methods of assessing protein quality are open to criticism, particularly if the data are to be extrapolated to the human situation, but nevertheless, the results presented in Table III give some cause for confidence that the yoghurt mixture could be usefully employed in a human diet. It was also possible to confirm that the product would be microbiologically acceptable, in that the total viable count was

TABLE II

Amino-acid composition of a yoghurt-cereal mixture, together with the provisional pattern suggested by FAO/WHO (1973); (g/100 g of protein.) Published data for skim-milk powder and wheat flour are included for comparison.

Amino-acid (Essential)	Yoghurt-cereal mixture	Whole wheat flour[a]	FAO/WHO standard	Skim-milk powder[b]
Isoleucine	4.8	3.8	4.0	6.7
Leucine	8.4	6.4	7.0	10.4
Lysine	4.4	2.7	5.5	8.0
Methionine + Cysteine	5.7	3.7	3.5	2.2
Phenylalanine + Tyrosine	8.0	7.8	6.0	4.4
Threonine	3.0	2.9	4.0	4.8
Tryptophan	1.1	1.3	1.0	1.4
Valine	5.2	4.3	5.0	6.9

Source.
[a]Kent (1970)
[b]Jones (1974)

TABLE III

Estimation of the Protein Efficiency Ratio (PER) and Net Protein Utilisation (NPU) of a yoghurt-cereal mixture. All values are expressed as means ± standard error, and each diet-group consisted of ten animals.

Source of protein	PER	NPU
Casein	2.5 ± 0.6	62.0 ± 5.8
Yoghurt-wheat mixture	2.3 ± 0.3	61.2 ± 7.0

below 10,000 colony forming units per gram of product. Coliform organisms were not detected in any of the samples examined, and yeasts and moulds were also absent. The hygienic quality of the product was, therefore, rated as satisfactory, and it was possible to proceed with the field trials aimed at gauging the overall acceptability of the material as judged by consumer reaction.

SENSORY APPRAISAL

A cursory examination of the food habits of rural communities in Mexico indicated that extensive use was made of a grüel called *atole*. This atole is a hot drink prepared from flour and water, with sugar added to make it more appealing, and it is widely consumed at breakfast and/or dinner time by children. It seemed reasonable to propose, therefore, that, if the rice or maize flour base of atole could be replaced with the yoghurt-cereal mixture, then the nutritional value of the atole could be elevated to a more desirable level. It was also important that children find the native gruels attractive, because it was considered that this group was the sector at most risk from malnutrition. However, acceptability of a food by a child is of little value if the mother does not consider it appropriate, and hence the initial introduction was made through the parents.

The first stage of the study was, of course, to establish a pattern of consumption for the traditional gruels, and, for this survey, a rural area at Malinalco, Estado de Mexico was selected. Some 40 families were chosen on the basis that at least some of their children were between 0–6 years,

TABLE IV

A summary of the questions asked of forty families in the community of Malinalco, Mexico, concerning the use of cereal gruels (*atole*), and the responses received.

Question				
Do you give atole to your children?				
Response	YES	NO		
	75%	25%		
At what age were your children when you started giving atole to them?[a]				
Response	0–3 months	3–6	6–12	after 12 months
	5%	30%	50%	15%
What flour do you usually use to make the gruel?				
Response	Rice	Maize	Wheat	Other
	58%	23%	10%	9%
After tasting the cups of atole provided, arrange them in order of preference.[b]				
A) First—most liked				
B) Second				
C) Third				
D) Fourth—least liked				
Response	Strawberry	Cinnamon	Vanilla	Natural
Adults	(A)	(B)	(C)	(D)
Children	(A)	(B)	(C)	(D)

[a]Only asked of mothers who gave atole to their children.
[b]Asked of all forty mothers, and twenty-four children.

and the mothers in each family were asked the questions set out in Table IV.

The results of this survey indicated quite clearly that, as anticipated, gruels do form an important part of the rural Mexican diet, particularly for young children. How frequently the gruels were consumed, or in what quantity, was beyond the scope of this survey, but it was estimated that most children drank between 400–800 ml of gruel per day. If this estimate is accurate, then it would seem that an appreciable portion of the food intake of children around one year comes from the atole, and hence there would be some merit in raising its nutritional value. The figures in Table V tend to confirm that the introduction of gruels based on yoghurt mixtures would have this effect, for although the protein content of the commercial mixtures has been improved by the addition of soya flour, the milk-based product would still offer certain advantages. Nevertheless, the yoghurt-cereal gruel has also to prove acceptable in other respects, and it is of note that the preference for rice flour stems, in part, from the fact that it is more readily dispersed in water than the maize flour, and hence is regarded as being easier to digest; it was important, therefore, for the yoghurt-cereal gruel to be similar in consistency with its rice counterpart. The other feature of consumption that was

TABLE V

Comparative dietary intakes of protein, carbohydrate and fat from gruels made of either cereal flours, commercial *atole* mixes or a yoghurt-cereal mixture. It is assumed that 500 ml of *atole* are consumed with a total solids of 5.6 percent.

Base material	Protein	Carbohydrate (g/500 ml)	Fat
Maize meal[a]	2.6	20.1	1.1
Maize starch[a]	0.1	24.3	0.08
Rice flour[a]	1.9	22.4	0.2
Wheat flour[a]	3.1	21.8	0.4
Commercial mix (A)	3.9	21.8	1.2
Commercial mix (B)	3.9	21.8	0.5
Yoghurt-cereal mixture	4.8	20.0	0.5

[a]Source—Scade (1975).

pertinent to the present study was that the gruels were made with milk if the latter was available, and this desire to incorporate milk into the diet suggested that a gruel based on yoghurt would be well received by the mothers.

It was against this background that the acceptability trial with gruels made from the yoghurt-cereal mixture was carried out. The test gruels were made by dispersing 56 g of the dry mixture in 1000 ml of tepid water, and then warming the suspensions up to boiling. Sugar was added at a five percent level, and three flavours were prepared as alternatives, namely strawberry, vanilla and cinnamon. Samples of these three gruels, together with an unflavoured sample, were then presented to those mothers and children that were familiar with atole, even though some of the families did not consume atole in their own homes (see Table IV). Thus 40 mothers and 24 children (approximate age range 3–5 years) took part in the trial, and, for simplicity, mothers and children were asked only to place the samples in order of preference. On this basis, strawberry emerged as the clear favourite, with vanilla, cinnamon and natural following in sequence. Whether colour or flavour was the strongest influence in the selection of strawberry could not be ascertained, but the rejection of the natural flavour was somewhat disappointing. Thus, although the plain yoghurt-cereal mixture would normally be in competition only with the local rice or maize gruels, it would have been encouraging if the natural yoghurt mixture had been better received. Nevertheless, most mothers indicated that they would buy the natural yoghurt-cereal mixture if it was available in their local shops, and this reaction alone may indicate that acceptability of the new product would not pose too many problems.

DISCUSSION

When a milk product, like yoghurt, is preserved by drying in combination with a cereal flour, it is hardly surprising that the nutritive value of the product is higher than that of the flour alone. It would seem appropriate, therefore, to suggest that, in those countries where cereals are the staple diet and milk is in limited supply, more attention should be given to the idea of producing yoghurt-cereal mixtures for consumer-use.

The validity of this view has been confirmed, at least in part, by the present study, in that it has shown first that yoghurt-cereal mixtures could, in nutritional terms, form a valuable supplement to a carbohydrate-rich diet; second that the product could be produced with a modest level of technology; third that the product should present no health hazard to a potential consumer.

These conclusions are in accord with the views of van Veen and Steinkraus (1970), but it is important that this present study has also considered in some detail the problem of acceptability. Thus, the consumption of yoghurt-cereal mixtures is rarely encountered outside the Middle East, even though many countries face similar problems of milk distribution and storage. Obviously many factors contribute to the evolution of food habits, but it is rather curious that the simple process of drying fermented milks with cereal flours has not spread more widely. The eccentricity of this restricted distribution is highlighted by the fact that the yoghurt mixture produced during this present investigation was readily accepted by a typical rural community in Mexico. It must be admitted that the mixture was presented in the form of a familiar gruel, atole, and with careful attention to flavour and consistency, but nevertheless, even the natural product was a accepted by most participants in the trial.

The possible value of this latter observation is that it confirms not only that yoghurt-based products could enjoy more widespread popularity, but, furthermore, that they could provide a vehicle for introducing valuable milk protein into the diet of underprivileged children. Thus, many children in rural Mexico consume, even at an early age, around 500 ml of atole per day, so that the substitution of cereal flour with a yoghurt-based material must be beneficial. If, in addition, alternative carriers for the yoghurt mixture could be found, then it is feasible to propose that yoghurt-cereal flours might go some way to alleviating child malnutrition in the rural areas of Mexico and/or similar communities.

If it is accepted that these mixtures would be welcome in rural communities, then their production offers a potentially valuable method of conserving the erratic milk supplies that characterise the dairy activities in many developing countries. The exact method of exploiting this approach to food conservation will vary from one country to another, but it is probable that at least some losses of liquid milk could be halted if the spread of this particular fermentation process could be encouraged.

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IMPROVING THE IMAGE OF YOGHURT-CEREAL MIXTURES

by

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ABSTRACT

Fermented yoghurt-cereal mixtures enjoy widespread usage in the Middle East, where they are employed as a means of preserving valuable supplies of milk protein. However, the unusual nature and flavour of these local products gives them a rather restricted appeal, and it appears that these singular characteristics result from the uncontrolled nature of the fermentation. Thus, it has been shown that when the yoghurt flora is limited to *Streptococcus thermophilus* and *Lactobacillus bulgaricus,* and the second-stage yoghurt-cereal fermentation is carried out under conditions which also favour the inoculated bacteria, then a mild, agreeable flavour is imparted to the end-product.

Various other processing features, such as a more complete grinding of the wheat, and a rapid termination of the second fermentation, also improve the nature of the finished material. It is proposed, therefore, that this more uniform product could be promoted as a valuable food supplement for developing countries, and perhaps also as an attractive breakfast cereal for populations in urban areas.

Although the lactic fermentation of milk has much to recommend it is as a means of both preserving the raw material, and producing an organoleptically attractive end-product, the shelf-life of yoghurt or similar foodstuffs is still limited. This latter problem is highlighted in some countries of the Middle East where the high ambient temperatures pose a real limitation on storage, and the most practical solution has been found to involve 'drying' the yoghurt with cereal flours.

A number of variants of the basic yoghurt-cereal mixture are available according to local custom, but the end-products share a number of essential features in that:

1) they form a valuable dietary ingredient, particularly during periods of the year when milk production is low;

2) their non-hygroscopic nature, together with a low pH, makes it possible to store them at ambient temperature with few problems.

However, despite these obviously desirable characteristics, the exploitation of materials of this type is, outside certain areas, minimal. One of the reasons for this lack of enthusiasm may be linked with the absence of 'consumer appeal' evident with some of the products made at village level. Thus, in general they are extremely acid, up to 2.5% lactic acid, have a rather acrid flavour, and, when reconstituted to form a 'porridge-like' gruel (the normal method of consumption), the resultant mixture has a granular consistency that is disagreeable to many palates.

Nevertheless, the basic concept is an attractive one, and it was decided, therefore, that an attempt should be made to both standardise the process, and to modify it in such a way as to give the end-product a more 'universal' appeal. If this could be done, then it was hoped that other populations, especially those with erratic milk supplies, might be tempted to look at yoghurt-cereal mixtures in a new light.

Traditional yoghurt-cereal mixtures

One of the best known of these materials 'kishk' is already widely consumed in the rural areas of Iraq (Frankul & Pellet, 1959), while a similar product 'Tarhana' is to be found in Turkey (Koksal, 1961).

The basic process starts with the treatment of the cereal, normally wheat, and, after washing to remove any foreign materials, the grain is boiled for an hour or so in open kettles. This process gelatinizes the starch (Neufeld *et al,* 1957), so altering its solubility through the formation of amylose and amylopectin complexes (Priestly, 1976 (a) & (b), 1977). The parboiled grain is then spread on the ground to dry, and, being a mid-summer activity, the sun rapidly lowers the moisture content to around 12%. After removing the chaff, the grain is then milled into a coarse flour ready for storage.

The final stages of producing 'kishk' involve mixing the ground, parboiled wheat into concentrated yoghurt (Robinson, 1977), and allowing the 'dough-like' mix to ferment for up to five days. What microbiological processes are involved in this stage are still largely unknown, but traditionally, however, the fermentation

is allowed to run its natural course, and the finished dough is then turned into small rolls, about 70-80 cm in diameter, and sun dried.

Although there are some losses of vitamins from the raw materials during processing (Saracoglu, 1953, Adolph *et al,* 1955), the protein content of the dry-material is around 24%, and it is estimated that 50 g. of 'Kishk' per day can meet the lysine requirements of a man 60 Kg body weight. Nevertheless, in spite of these attractive attributes of the product, the lack of 'process control' results in a material that is both variable, and of such an 'acquired' taste, that it is frequently unpalatable to those unfamiliar with the flavour. It was these latter features that the laboratory process sought to eliminate.

The Suggested Process

The cereal portion of the mixture was prepared by soaking wheat (var. Mega) at 60°C for four hours, and, after draining off the water, 'steaming' it under pressure (15 psi) for 10 minutes. The grain was then dried at 50°C to 10% moisture and milled; the flour and bran were mixed together to give the final ingredient.

The yoghurt fraction was made with reconstituted skim-milk (12% total solids) which was heat-treated at 85°C for 30 minutes prior to inoculation. The starter culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was added at a rate of 2% to the cooled milk (45°C), and the mixture was incubated at 42°C for 4 hours; the acidity of the yoghurt at this point was 1.5% lactic acid (1.a.)

The yoghurt was then added to an equal volume of a prepared flour-water mixture (1 part whole flour: 2 parts water), and the whole was agitated to a homogeneous consistency. This 'dough' was allowed to ferment for 48 hours at 42°C, by which time the acidity of mixture, which had been lowered to 0.5% 1.a. by the flour, had risen to around 1.0% 1.a.. The fermentation was terminated by macerating the dough in an excess of hot water (80°C), and holding the suspension at this temperature for 30 minutes. After this time the mixture was roller-dried, and the resultant material was then lightly ground to give the finished product.

Results and Discussion

When the end-product was reconstituted with hot water, the resultant 'porridge' had a pleasant, malty flavour, and a consistency (variable according to personal taste) that was smooth on the palate. This contrast with the traditional 'Kishk' is, to a large extent, a reflection of the improved control over the fermentation stages, in that not only was the acidity much lower, but also the restricted microflora gave little opportunity for the development of 'off-flavours'.

A chemical analysis of the product revealed that its protein content was rather lower than the native material (see Table 1), but the employment of concentrated yoghurt for the village process could explain this difference.

It was also noticeable that the sample from Iraq contained a percentage of sodium chloride, and this optional addition (at a level in excess of 2%) is partly responsible for the elevated ash content. The vitamin B components are comparable with other reported analyses (Frankul, 1961, Van Veen *et al,* 1969), and it is probable that some processing losses, vis-a-vis the raw materials, are inevitable. However, the employment of raw flour, with its ungelatinised starch and harsh flavour, makes a product that is virtually inedible, while the vitamin depletion associated with the terminal drying/heating procedure cannot be readily avoided. The replacement of 20% of the 'normal' flour by flour from malted wheat was found to be one way of elevating the vitamin content, but whether there is any nutritional justification for such a practice must be open to doubt.

Obviously chemical analyses alone do not support firm conclusions concerning the nutritional value of a foodstuff, but there is every reason to believe that the 'new' yoghurt-cereal mixture would be no less acceptable a dietary component than its native counterpart (van Veen & Steinkraus, 1970). If this view is correct, then the new process would offer a product that, in addition to the attributes of the original 'Kishk', could be expected to:

1) be reasonably uniform from one batch to the next;
2) have a mild, malty flavour that could well find widespread acceptance, although it is possible that the acidity would have to be raised slightly to combat adverse storage conditions;
3) be produced with a comparatively low level of technology.

It is reasonable to conclude, therefore, that a yoghurt-cereal mixture of the type described could be considered for utilisation by peoples with entirely different food patterns and preferences from those in the Middle East. Furthermore, not only might the product

TABLE 1 Composition of a laboratory-made yoghurt-cereal mixture, together with a village-made sample from Iraq.

Product	Protein (g/100g)	Ash	Niacin (mg/100g)	Riboflavin	Thiamin
Laboratory origin	18.2	2.33	3.22	0.31	0.13
Origin in Iraq	29.4	11.6	2.87	0.41	0.06

(After Cadena, 1977).

prove suitable for exploitation as a means of preserving valuable sources of milk protein in rural communities, but also, perhaps, as an attractive breakfast food for urban dwellers. Thus, the trend towards 'natural' breakfast cereals has been quite marked in recent years, and consumer reaction to a yoghurt-based product could prove interesting.

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BOEKBESPREKING

LABORATORY METHODS IN FOOD AND DAIRY MICROBIOLOGY

by W F Harrigan and Margaret E McCance. Hersien deur W F Harrigan, 1976. Academic Press, London. 452 pp. Plaaslike prys ongeveer R 23.

Hierdie is 'n hiersiene uitgawe van die oorspronklike boek met die titel *Laboratory methods in microbiology*. Die verandering in die titel van die book beklemtoon in 'n mate die huidige neiging tot die samesmelting van die suiwelbedryf met die breëre voedselbedryf.

Die inhoud van die boek is gereorganiseer en aangevul. Dit bestaan uit drie dele en vier bylaes. Die eerste deel handel oor basiese metodes; die tweede oor tegnieke in die toegepaste mikrobiologie, terwyl die derde deel skemas bevat vir die identifikasie van mikroörganismes. Die bylaes bevat resepte vir kleurstowwe en kweekbodems, tabelle vir berekening van die "mees waarskynlike getal", 'n lys van fabrikante en leweransiers van mikrobiologiese benodigdhede, 'n geselekteerde bibliografie en lys van verwysings.

Die beskrywing van die verskillende toetsmetodes word voorafgegaan deur inleidende paragrawe waarvan die inhoud baie help met die vertolking van resultate verkry met mikrobiologiese ontledings van voedselprodukte.

Baie mikrobioloë is onkundig omtrent die gevare verbonde aan die chemikalieë en materiaal waarmee hulle werk aangesien die meeste mikrobiologiehandboeke min melding hiervan maak. Gevolglik is die gedeeltes oor veiligheidsmaatreëls en die gevare verbonde aan die hantering van sekere chemikalië nuttige toevoegings tot die boek.

Hierdie boek kan sterk aanbeveel word vir studente in die voedsel- en suiwelmikrobiologie en vir persone wat betrokke is by die mikrobiologiese kwaliteitskontrole van voedsel- en suiwelprodukte.

B H Bester

OLD SOUTH AFRICAN DAIRY JOURNALS

The Animal and Dairy Science Research Institute is anxious to include in their collection of dairy literature old South African dairy literature. It will be greatly appreciated if anyone could give any information as to where the journals listed below, could be obtained or those who are prepared to make a contribution to the library. Please write to the editor, P.O. Box 103, Irene, 1675.

Journal	Approximate years of publication
1. South African Dairy Journal	1936, 1937
2. South African Milk Journal	1936, 1937
3. South African Dairyman	1939 to 1959
4. South African Dairyman and Smallholder	? to 1959
5. Fluid Milk	1939 to 1959
6. South African Smallholder and Dairyman	? to 1959
7. Dairy Farmer	1948
8. Farm illustrated News (Dairying)	1948 to 1953
9. South African Dairy Industries Journal	1947 to 1948
10. South African Dairy Farmer	1946 to 1947

ELSEVIER

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Food Quality and Preference

Some nutritional and sensory properties of bulgur and whole wheatmeal kishk (a fermented milk-wheat mixture)

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Abstract

Changes in phytic acid, phytase activity and HCl-extractability of Ca, Fe, Mg and Zn during fermentation of kishk formulated from bulgur (cracked and bran-free parboiled wheat) or whole wheatmeal were monitored. Phytic acid and phytase activity decreased and the proportions of HCl-extractable Ca, Fe, Mg and Zn increased as fermentation progressed. The whole wheatmeal kishk contained lower ($p < 0.05$) amounts of phytic acid and its Ca, Fe, Mg and Zn were more ($p < 0.05$) amenable to extraction by 0.03 N HCl than bulgur kishk. The soup prepared from whole wheatmeal kishk was significantly ($p < 0.05$) more yellowish in colour, more sour, less gritty, less cohesive and contained more bran particles than the bulgur-based formulation. Both formulations were liked to a similar degree. These findings suggest that substitution of whole wheatmeal for bulgur in the formulation of kishk enhances the availability of Ca, Fe, Mg and Zn without undue effects on the acceptability of the final product.

1. Introduction

Fermented milk-wheat mixtures, known as kishk in the Middle East and tarhana in Greece and Turkey, are important items in the diet of many populations. In addition to their well-established position in the dietary patterns of the people in the aforementioned countries, these products have been promoted in Mexico (Cadena & Robinson, 1979) and Europe (Berghofer, 1987). Kishk is typically prepared by adding yoghurt/strained yoghurt to bulgur (cracked and bran-free parboiled wheat) and allowing the mix to ferment at ambient temperature for different periods of time. The resulting paste is dried to a moisture content of ca. 10–13% and then ground into a fine powder. The powder is reconstituted with water, boiled and served as a soup. The processing, composition and sensory properties of kishk have been the subject of a recent review (Tamime & O'Connor, 1995).

Apart from being labour-intensive, the conversion of wheat into bulgur entails wastage of nutrients especially as only 84–86% of the wheat is normally recovered as bulgur (Williams & El-Haramein, 1984). Therefore, the substitution of whole wheatmeal for bulgur in the formulation of kishk should provide a better means for the utilization of wheat nutrients. However, in addition to any impact on the sensory properties of the final product, the substitution might be expected to reduce the availability of minerals in view of the presence of high concentrations of phytic acid in the bran fraction of wheat (Reddy, Sathe, & Salunke, 1982; Lehrfeld and Wu, 1991).

Consequently, the objectives of the present study are to: (1) determine the effects of substituting whole wheatmeal for bulgur in the formulation of kishk on the HCl-extractability of Ca, Fe, Mg and Zn; the HCl-extractability of minerals is considered as an index of their availability to humans by different workers (Chompreeda & Fields, 1984; Mahajan & Chauhan, 1988; Kumar & Chauhan, 1993); (2) relate the changes in the HCl-extractability to the levels of phytic acid and apparent phytase activity during the fermentation; and (3) assess the changes in sensory properties and acceptability of the final products brought about by the indicated substitution.

2. Materials and methods

2.1. Materials

Cows (Holstein) milk and wheat (Najah) were obtained from the Agricultural Research and Education Center of the American University of Beirut. Freeze-dried starter cultures of *Streptococcus thermophilus* and

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Lactobacillus delbrueckii subsp. bulgaricus (1:1 mixture) were obtained from Chr. Hansen Laboratories (Horshölm, Denmark). Coarse salt was purchased from the local market. The reagents used for the chemical analyses were of Analytical Grade.

2.2. Methods

2.2.1. Preparation of yoghurt and strained yoghurt (Labneh)

Milk (20 l) was heated in a steam-jacketed stainless steel vessel at 85°C for 20 min, cooled to 42°C and inoculated with a 2% active starter culture propagated from a lyophilized 1:1 mixture of *Str. thermophilus* and *Lb. bulgaricus* as described by Tamime and Robinson (1985). The mixture was incubated (Precision Model GM, GCA Corporation, IL., USA) at 42°C until a pH of 4.5–4.6 was attained (ca. 3–4 h).

Strained yoghurt (labneh) was prepared by placing yoghurt in a cloth bag and allowing the mix to drain by gravity, at 4–6°C, until a total solids content of 24–26% was attained (ca. 4–5 h).

2.2.2. Preparation of bulgur and whole wheatmeal

Bulgur was prepared as described by Toufeili, Olabi, Shadarevian, Abiantoun, Zurayk, & Baalbaki, (1997). Wheat kernels, cleaned from extraneous matter, were steeped in water (wheat:water; 2:3) for 30 min and then boiled until the water has been absorbed (ca. 80–90 min). The cooked kernels were dried in a cabinet drier (Hotpack, Philadelphia, USA) until a moisture content of 7–8 g per 100 g was attained (ca. 12–16 h). The dried kernels were milled in a Wiley mill (Arthur Thomas, Philadelphia, USA) fitted with a 5.5 mm screen and the ground material was freed from bran by air elutriation using a fan; the mesh size was selected to represent normal commercial practice. The bulgur particles were placed in polyethylene bags and stored at 5°C until used.

Whole wheatmeal was prepared by milling the dried parboiled wheat grains in an Alpine mill (Model 160Z, Augsburg, Germany) fitted with 2.5 mm screen. The milled samples were stored in sealed polyethylene bags, at 5°C, until used.

2.2.3. Preparation of kishk samples

Kishk was prepared as described by Sabra (1994). The cereal ingredient (bulgur or whole wheatmeal) was heated at 100°C for 15 min and then cooled to room temperature to reduce its microbial load and to improve the flavour of the final product. Yoghurt (1 kg) and coarse salt (30 g) was added to a batch of bulgur or whole wheatmeal (500 g) and the resulting mixture was kneaded in a Crypto dough mixer (Model E.B.12, Crypto Ltd, London), at low speed, for 5 min. A sample was freeze-dried (Stokes, Model 902-1-8, Penwalt Corporation, Philadelphia, USA) for future analysis and the rest was incubated in closed earthenware containers at 35°C for 24 h. Labneh (800 g) was added and the mixture kneaded as described previously. Again one sample was freeze-dried and the rest was incubated, in closed earthenware containers, at 35°C for 72 h. At the end of the fermentation, the containers were removed from the incubator, a sample was freeze-dried and the bulk was formed into small balls of 3–4 cm in diameter and dried in a cabinet drier at 55°C (ca. 18–22 h). The resulting dry product was ground in an Alpine mill fitted with 2.5 mm screen and stored in polyethylene bags, at 5°C, until used. Duplicate preparations were made for each type of kishk.

2.2.4. Chemical analyses

Moisture, protein, fat and ash contents were determined, in triplicate, according to Association of Official Analytical Chemists (1990) methods 925.23, 925.105, 920.125 and 945.46, respectively.

Total and HCl-extractable Ca, Fe, Mg and Zn were determined as outlined by Chompreeda and Fields (1984). The minerals were extracted with 0.03 N HCl at 37°C for 3 h and the extract filtered and dried on a hot plate (80°C). The samples and the dried HCl-extracts were wet ashed in a mixture of nitric and perchloric acids and the Ca, Fe, Mg and Zn contents determined by atomic absorption spectrophotometry (GBC double beam, Model 902, GBC Scientific Equipment, Victoria, Australia). A Sample of known mineral content (whole milk powder, RM 8435, National Institute of Standards and Technology, MD, USA) was assayed at the same time. The HCl extractability is expressed as: minerals extractable in 0.03 N HCl × 100/ total minerals.

Phytic acid was determined as described by Khokhar, Pushpanjali, and Fenwick, (1994); apparent phytase activity was measured at 35°C as outlined by Kilmer, Seib, and Hoseney, (1994); the determinations of HCl-extractability, phytic acid and apparent phytase activity were conducted in triplicate on the duplicate preparations of kishk.

2.2.5. Sensory evaluation

Nine panelists (4 males, 5 females, age 23–30 yr), identified as consumers of kishk and who had completed a graduate course in sensory analysis, participated in the study. The panelists were trained in the sensory profiling of kishk for 8 one-hour sessions over a period of 4 weeks. Seven commercial samples of kishk were used in the training. The panelists used group discussions to establish descriptive terms characterising the flavour and texture attributes of kishk. The attributes selected were: colour, presence of bran particles, sourness, saltiness, grittiness, cohesiveness, moisture absorption and astringency. Definitions of the indicated attributes are presented in Table 1. Consistency of the panel was

checked by cluster analysis (Ward's method) of four replicate ratings of the 7 commercial kishk samples for the indicated attributes (Malundo & Resurreccion, 1992).

The attributes' descriptor ranges were: colour (creamy white–yellow), presence of bran particles (none–many), sourness (absent–very strong), saltiness (absent–very strong), grittiness (smooth–very gritty), cohesiveness (loose mass–cohesive mass), moisture absorption (no absorption–large amount of absorption) and astringency (absent–very strong).

The sensory score sheet consisted of 15-cm unstructured line scales anchored at the extremes with the appropriate descriptors. In evaluating the samples, panelists were instructed to place a vertical line across the horizontal line at the point that best quantified their evaluation of intensity. The magnitude of the responses was scaled by measuring the distance from zero to the position of the vertical line.

Soups were prepared by adding 20 g dried kishk to 170 ml of water and heating with gentle stirring to boiling, simmering for 7 min and cooling to 40°C. The samples (20 g) were placed in identical glass containers (8 cm diameter, 3.5 cm height) and served at 40°C. Two kishk samples (one whole wheatmeal and one bulgur kishk) were presented simultaneously, in a balanced random order, to the panelists and rated for the indicated attributes, in a session. The assessments were replicated three times with one session being conducted in the morning, at the first day, and two sessions, with an interval of 1 hr between sessions, in the afternoon, on the next day. Sensory assessments were, thus, completed over a period of 2 days. The samples were coded with 3-digit codes and assessed in partitioned booths equipped with white light. The assessors were asked to rinse their mouths with water between samples.

The acceptability of the samples was rated on a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) by 50 subjects (age 19–59 years) from the students, faculty and staff of the Faculty of Agricultural and Food Sciences at the American University of Beirut. The logistics of the test (sample size, container, serving temperature, coded samples, order of presentation) were as described previously.

3. STATISTICAL ANALYSES

Differences in phytic acid levels, apparent phytase activity and HCl-extractability of minerals between the two types of kishk were ascertained by *t*-tests (Gacula & Singh, 1984). Sensory scores were subjected to analysis of variance (ANOVA) of a hierarchical classification of data to ascertain differences between type of kishk and preparation (Steel & Torrie, 1982). The repeatability of determinations was estimated by calculating a least significant difference between replicates after ANOVA of a nested classification of data (Caulcutt & Boddy, 1983). The analyses of variance and *t*-tests were carried out using the MSTAT (1989) programs hierarch and *t*-test.

4. RESULTS AND DISCUSSION

The composition and amounts of total and HCl-extractable Ca, Fe, Mg and Zn of the ingredients used in the preparation of kishk are shown in Tables 2 and 3.

4.1. Phytic acid and phytase

The bulgur-based formulation contained more phytic acid ($p < 0.05$) throughout the fermentation, as compared to its whole wheatmeal counterpart (Table 4), in spite of the presence of the bulk of phytic acid in the bran fraction of wheat (Lehrfeld & Wu, 1991). This apparent anomaly could be attributed to the migration of phytic acid from the bran to the inner parts of the kernel upon parboiling especially as the phytic acid contents of the bulgur and the bran separated therefrom were found to be 13.12 mg g^{-1} and 6.30 mg g^{-1}, respectively. The migration of nutrients from the bran to the interior of the grain upon parboiling has been reported in wheat (Sabry and Tannous, 1961) and rice (Bhattacharya & Ali, 1985).

A marked decrease in the phytic acid content of the formulations was observed during the first 24 h of fermentation with 25.5% and 44.47% of the original phytic acid content being hydrolysed in the bulgur and whole wheatmeal-based formulations, respectively. Lesser changes in the phytic acid levels of the formulations

Table 1
Definitions of sensory attributes selected for evaluation of kishk

Attribute	Definition
Colour	The actual colour or hue of the product, for example, yellow.
Presence of bran particles	The relative number/amount of brown specks (bran particles) present on the surface of sample.
Sourness	The taste stimulated by, for example, citric acid.
Saltiness	The taste stimulated by, for example, sodium chloride.
Astringency	The puckering of the tongue surface caused by, for example, tannins.
Grittiness	The sensation elicited by teeth due to the hard particles present, for example, in pear stone cells.
Cohesiveness	The degree to which sample holds together upon manipulation between tongue and palate.
Moisture absorption	The amount of saliva absorbed by sample during manipulation in the oral cavity.

Table 2
Composition[a] of yoghurt, labneh, bulgur and whole wheat

Product	Protein (g per 100 g)	Fat (g per 100 g)	Ash (g per 100 g)	Moisture (g per 100 g)	Nitrogen-free extract[b] (g per 100 g)
Yoghurt	3.04±0.01[c]	3.6±0.11	0.56±0.01	87.7±0.26	5.08±0.13
Labneh	8.86±0.06[c]	10.4±0.33	0.83±00.2	75.5±0.21	4.37±0.34
Bulgur	14.72±0.22[d]	1.19±0.01	1.65±0.01	8.12±0.32	74.32±0.57
Whole wheat	14.61±0.12[d]	1.46±0.01	1.83±0.03	7.20±0.18	74.90±0.23

[a] Average of triplicate determinations ± SEM.
[b] By difference.
[c] N × 6.38.
[d] N × 5.70.

Table 3
Amount[a] of total (mg per 100 g on dry mass basis) and HCl-extractable (%) calcium, magnesium, iron and zinc of yoghurt, labneh, bulgur and whole wheat

	Calcium		Magnesium		Iron		Zinc	
	Total	HCl-Extractable	Total	HCl-Extractable	Total	HCl-Extractable	Total	HCl-Extractable
Yoghurt	795.8±3.85	86.3±0.88	46.3±0.67	66.0±1.53	1.6±0.12	89.1±4.22	2.1±0.05	86.3±0.83
Labneh	498.2±9.50	84.0±0.99	28.7±0.16	80.6±3.48	2.7±0.07	90.4±3.8	3.0±0.06	92.8±0.93
Bulgur	77.1±0.28	77.6±0.88	211.6±4.21	56.6±3.33	8.4±0.27	46.3±0.59	2.9±0.06	28.0±0.34
Whole wheat	69.4±0.73	75.6±0.88	182.8±3.76	53.6±3.17	8.8±0.07	39.4±0.17	2.3±0.03	20.2±0.33

[a] Average of triplicate determinations ± SEM.

Table 4
Changes in phytic acid content and phytase activity in bulgur kishk and whole wheat kishk during fermentation

	Phytic acid[a] (mg per 100 g)		Phytase[a] (mmol Pi g^{-1} h^{-1})	
	Bulgur kishk[b]	Whole wheat kishk[b]	Bulgur kishk[b]	Whole wheat kishk[b]
Time (h)				
0	855.58	730.41	1.83	4.02
24	637.49	405.62	0.29	0.64
96	520.00	293.18	0.15	0.32
SEM[c]	14.09		0.06	
LSD[d]	41.12		0.18	

[a] Average of triplicate determinations on two replicate preparations.
[b] Significant differences between bulgur kishk and whole wheat kishk were detected throughout the fermentation by t-test ($p < 0.05$).
[c] Standard error of mean.
[d] Least significant difference ($\alpha < 0.05$).

were observed afterwards. Similar findings on the decrease of phytic acid have been reported upon natural fermentation of a range of cereals, legumes and tubers (Marfo, Simpson, Iodwin, & Oke, 1990), corn (Lopez, Gordon, & Fields 1983), milk-wheat mixtures (Gupta & Khetarpaul, 1993), brown beans (Gustafsson & Sandberg, 1995) and lentils (Kozlowska, Honke, Sadowska, Frias, & Vidal-Vafuerde, 1996).

The reduction in the phytic acid content of cereals and legumes upon fermentation has been attributed to the activity of the indigenous phytases of the constituent ingredients and/or those elaborated by the fermenting microorganisms (Reddy et al., 1982; Dhanker & Chauhan, 1987; Sandberg & Svanberg, 1991). In the present study, apparent phytase activity was detected only in the cereal, bulgur or whole wheatmeal, ingredient of the formulation (data not shown). In addition, the apparent phytase activity decreased sharply upon fermentation thus suggesting little if any microbial phytase activity. These observations point to the dominant impact of wheat phytases in the hydrolysis of phytic acid during the course of the fermentation.

The higher apparent phytase activity of the whole wheatmeal-based formulation is consistent with the presence of the bulk of the phytase activity in the aleurone layer of wheat (Peers, 1953; Lim & Tate, 1973).

The survival of phytase during the boiling step in the preparation of bulgur and whole wheatmeal (refer to the Materials and Methods section) is in accord with the observations of Ranhotra and Loewe (1975) who reported only 25% loss in the original activity of wheat phytase after heating for 1.5 h at 100°C. The relatively slow degradation of phytic acid after 24 h of fermentation could be attributed to the inhibition of phytase by both inorganic phosphates released upon the hydrolysis of phytic acid (Ranhotra, Loewe, & Puyat, 1974) and organic acids produced through the metabolism of carbohydrates by the fermenting microorganisms (Marfo et al., 1990).

4.2. HCl-extractability of minerals

The total Ca, Mg, Fe and Zn, in mg per 100 g, in the bulgur and whole wheat kishks were 285.58, 116.83, 4.79, 2.51 and 281.17, 107.25, 4.96, 2.44, respectively.

The HCl-extractability of minerals increased as fermentation progressed (Table 5). This increase could be attributed, at least in part, to the decrease in phytic acid levels observed during the fermentation (Table 4). Significant negative correlations between phytic acid and availability of minerals have been reported (Gupta & Khetarpaul, 1993; Saha, Weaver, & Mason 1994). Marked differences in the HCl-extractability patterns of the minerals were noted with Ca showing high and relatively little changes in HCl-extractability as fermentation progressed. This behaviour is presumably due to the low pH, ranging from 4.2 to 4.7, of the formulations; calcium has been reported to be 100% soluble at pH < 5 due to its inability to form Ca-phytate complexes irrespective of the concentration of phytic acid (Martin & Evans, 1986; Nolan, Duffin, & McWeeny, 1987).

Significant differences ($p < 0.05$) were noted in the levels of HCl-extractable Ca, Fe, Mg and Zn of the final products. The higher HCl-extractability of Ca, Fe, Mg and Zn from the whole wheatmeal-based product is consistent with the more extensive hydrolysis of phytic acid observed in this product as compared to its bulgur-based counterpart. These observations point to a potentially superior nutritional value for the whole wheatmeal formulation as compared to the traditional bulgur kishk.

4.3. Sensory evaluation

The mean scores of the sensory attributes of the two kishk formulations are presented in Table 6.

Significant differences ($p < 0.05$) were observed in the colour, presence of bran particles, sourness, grittiness and cohesiveness of the formulations. The whole wheatmeal formulation had a more yellowish tint, than the bulgur-based kishk, consistent with the presence of the bulk of the carotenoid pigments in the aleurone of the wheat kernel (Kruger & Reed, 1988). The whole wheatmeal kishk, understandably, contained more bran particles than the bulgur-based formulation. The higher degree of sourness detected in the soup prepared from whole wheatmeal kishk could be attributed to the relatively more efficient utilization of carbohydrates from the finely-divided matrix by the fermenting microorganisms, and the subsequent formation of higher concentrations of organic acids. The lower grittiness observed in the whole wheatmeal kishk, as compared to its bulgur-based counterpart, is due to the higher degree of size reduction employed in the preparation of whole wheatmeal flour. The relatively coarse and large-sized bulgur particles seemed to have structure-enhancing effects on the soup matrix as evidenced by the higher cohesiveness of the bulgur-based preparation.

No significant differences were detected in the preference of the formulations. Mean hedonic ratings of 6.24 ± 1.69 and 6.28 ± 2.00 were accorded to the bulgur and whole wheatmeal kishk, respectively. The similar mean ratings imply that both formulations were liked to a similar degree, but it was noteworthy that 20% of the assessors displayed a marked 'liking' or 'disliking' for one or other of the formulations.

Table 5
Changes in HCl-extractable[a] (%) calcium, magnesium, iron and zinc of bulgur kishk and whole wheat kishk during fermentation

Time (h)	Calcium		Magnesium		Iron		Zinc	
	Bulgur kishk	Whole wheat kishk	Bulgur kishk	Whole wheat kishk	Bulgur kishk	Whole wheat kishk	Bulgur kishk	Whole wheat kishk
0	86.3[b]	89.5[c]	39.1[b]	53.5[c]	31.8[b]	32.5[b]	35.2[b]	40.3[c]
24	87.8[b]	92.3[c]	73.0[b]	83.1[c]	39.1[b]	44.5[c]	45.0[b]	59.3[c]
96	89.1[b]	92.8[c]	72.8[b]	83.5[c]	43.8[b]	64.1[c]	72.2[b]	77.4[c]
SEM[d]	0.7		1.0		1.5		0.8	
LSD[e]	1.61		2.1		3.06		1.71	

[a] Average of triplicate determinations on two replicate preparations.
[b,c] Means with different superscripts, in a row, are significantly different by t-test ($p < 0.05$).
[d] Standard error of mean.
[e] Least significant difference ($\alpha < 0.05$).

Table 6
Mean Scores[a] of sensory attributes of bulgur kishk and whole wheat kishk

Attribute	Bulgur kishk	Whole wheat kishk	SEM[b]	LSD[c]
Color	5.5[d]	9.0[e]	0.5	1.40
Presence of bran	5.1[d]	9.3[e]	0.4	1.14
Sourness	6.6[d]	10.4[e]	0.5	1.21
Saltiness	7.6[d]	7.8[d]	0.5	1.29
Grittiness	10.1[d]	6.9[e]	0.4	1.00
Cohesiveness	8.5[d]	5.0[e]	0.5	1.31
Moisture absorption	7.9[d]	7.8[d]	0.7	1.81
Astringency	7.2[d]	6.9[d]	0.7	1.76

[a] Rated on 15-cm unstructured line scales. Average of 27 responses.
[b] Standard error of mean.
[c] Least significant difference ($\alpha < 0.05$).
[d,e] Means with different superscripts, in a row, are significantly different by t-test ($p < 0.05$).

5. Conclusions

Substitution of whole wheatmeal for bulgur in the formulation of kishk yielded a product with significantly ($p < 0.05$) less phytic acid and higher amounts of HCl-extractable Ca, Fe, Mg and Zn. Sensorily, the whole wheatmeal kishk is more sour, less cohesive, less gritty, contains more bran particles and is more yellowish in colour than the traditional bulgur kishk. In addition, the acceptability of this new type of kishk was similar to bulgur kishk. These findings coupled with the lower energy costs of production, point to the superiority of whole wheatmeal as an ingredient in the formulation of kishk.

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Research Article

HALLOUMI CHEESE - SOME ASPECTS OF MANUFACTURE AND QUALITY

by

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ABSTRACT

White-brined cheeses are extremely popular in both the Middle East and around the Mediterranean, and some types, such as Feta, are now manufactured in highly automated plants employing procedures that are fundamentally different from the traditional process. Another cheese in demand is Halloumi, but it has proved difficult to obtain the special textural properties of this variety using modern technology. This paper reviews some of the studies that have been carried out with a view to increasing the output and quality of this latter cheese, and highlights some possible avenues for further work.

Introduction

Halloumi is a semi-hard to hard, unripened cheese that is made from either sheep's or goat's milk or a mixture of the two (Robinson, 1991). The texture of the cheese is compact, but sliceable in thick slices, and its colour is white to yellowish; few holes or openings are present. When heated, Halloumi should melt evenly, have the capacity to stretch and have a tender mouthfeel. It is usually preserved in brine until delivered to the consumer, as are other similar cheeses like Feta, Akawi, Domiati and the boiled, brined cheese (Baladi Cheese) from Jordan.

Although primarily produced in Cyprus, it is widely popular in the Middle East, which, as an important market for milk products, is constantly seeking additional sources of supply, and of top quality. However, there is relatively little published information concerning the composition and/or methods of production of Halloumi cheese, and the aim of this paper is to review the available methods of manufacture, and suggest ways in which the process could be developed to meet the increasing demand.

Technology of Manufacture

Halloumi cheese is mainly produced from cow's or sheep's milk, but unless the manufacturing technique is adjusted, the cow's milk cheese is totally different from the ovine variety in terms of texture and other sensory properties; the bovine product often has a higher moisture content and, consequently, is less hard. The general procedure for manufacturing Halloumi is outlined in Figure 1, and the main differences between the processes employed for the two milks, as reported by Anifantakis and Kaminarides (1983), are as follows:

1. As sheep's milk has, typically, a total protein content of 5.6% as compared with 3.3% for cow's milk, the fat content of sheep's milk used for cheesemaking is standardised to 5.2% as against 3.1% for cow's milk; these adjustments provide a similar ratio of fat to protein in the final curd.
2. On cutting, the curd particles (sheep's milk) should be 1.0 cm^3 as compared with 0.5 cm^3 for the curd from cow's milk.
3. The curd from sheep's milk should be scalded at a lower temperature (40°C instead of 45°C), and with a shorter holding time of 20 minutes after the initial heating stage (15 minutes); with cow's milk, preliminary heating takes 20 minutes, and this stage is followed by a total 'hold' of 60 minutes - the final 20 minutes is accompanied by gentle stirring of the curd.
4. The initial pressing of the curd from sheep's milk is carried out at a lower pressure (3 kg/kg of curd) and for a shorter time (35 minutes) as compared with the bovine product (7 kg/kg of curd for 60 minutes).
5. The whey from sheep's milk is usually heated to 90 - 92°C, and the precipitated proteins are ladled into cheese cloths; to obtain a better precipitate and to improve the quality of the coagulum, a volume of milk equivalent to 10% of the original volume is added to the whey at 72°C. The whey cheese, known in Cyprus as Anarik, is sold as a fresh product on the following day; there is insufficient whey protein in cow's milk to obtain a comparable product.
6. Halloumi from sheep's milk is boiled in whey for around 30 minutes to achieve the desired texture, but the bovine product may take up to 60 minutes.

Dry salting, with or without the addition of dried leaves of mint *(Mentha viridis)*, is common to both varieties and, although connoisseurs prefer the ovine cheese, both types are acceptable to the majority of consumers.

As shown in Table I, the above procedures can give rise to cheeses of similar chemical composition but, with products from the market place, variability is a major

problem. Apart from the absence of facilities for standardisation, one reason may be that local producers rely entirely on the natural microflora to provide any ripening of the milk prior to renneting, and to enhance the complexity of flavour of the finished cheese. This traditional approach has been widely used in the past but, nowadays, makers of most cheese varieties prefer to add a selected starter culture to pasteurised milk (Tamime, 1990); this procedure allows both the process and the quality of the finished cheese to be more easily controlled.

It was with these latter points in mind that an investigation was carried out by Shaker *et al.* (1987) to produce Halloumi cheese using pasteurised cow's milk.

Fresh whole milk was pasteurised at 72°C for 15 sec., and then standardised using pasteurised skim-milk to give a casein/fat ratio of around 0.70. The temperature of the standardised milk was adjusted to 30°C, prior to inoculation with a culture of *Lactococcus lactis* sub-sp. *cremoris*. Calf rennet was stirred into the milk at a rate of 0.02%, and after around 40 minutes, the resultant curd was cut into cubes (1 cm^3). While being stirred, the contents of the vat were heated to 40°C over a period of around 10 minutes, and were held at this temperature for a short period. After draining-off the whey, the curd was pressed with increasing pressure for one hour. Meanwhile the whey was deproteinated by heating to 98°C and filtering through cheese-cloth. The pressed curd was then cut into pieces (10 x 15 x 3 cm) and heated in the whey. The textured pieces of curd were folded lengthways, allowed to cool for one hour and were then dry salted. After chilling overnight, the blocks were placed in brine (10% NaCl solution) for three hours and, after draining, were individually sealed into polythene bags. The end-product was rated fully comparable with traditional Halloumi.

The authors concluded that Halloumi cheese could be manufactured successfully from standardised cow's milk, and that the rate and degree of acid development was probably the most important process variable. In particular, as the level of acid development increased, so the lower values of pH and calcium/SNF ratios improved the melting and stretching characteristics of the heated cheese. Anifantakis and Kaminarides (1983) concluded also that processing conditions were critical with respect to product quality, and industrial-scale developments will clearly have to ensure a high degree of process control.

Chemical Composition and Microbiology of Halloumi

Some recorded compositions of Halloumi cheese are shown in Table I, (Antifantakis & Kaminarides, 1982, 1983) and it notable that sheep's milk cheeses from the market place in Cyprus vary widely from sample to sample. The high salt content of the traditional cheese was also evident, but whether the levels add to keeping quality is a moot point. In the traditional process, acidification of the milk takes place as a result of the growth of adventitious bacteria, and lactococci or lactobacilli might be expected to dominate. The extent to which thermoduric members of this microflora are involved with cheese maturation has yet to be established, but Anifantakis and Kaminarides (1985) showed that while freshly prepared Halloumi had a total microbial count of around 10^3 colony forming units (cfu) g-1 in the centre of the block, this figure rose to > 10^7 cfu g^{-1} over a period of two months at 4°C; at 20°C, the counts went even higher (see Table II), although numbers did appear to decline with age. Many of these bacteria will, of course, originate in the brine, and yeasts and coliforms found on the surface of the brined cheese are almost certainly casual contaminants; at levels above 1 000 cfu g^{-1}, yeasts can lead to a detectable deterioration in flavour (Haddadin, 1986). With on-farm production, these changes are uncontrolled, and yet even a limited move towards the use of starter cultures might allow a producer to manipulate, to some degree, the nature of the end-product. Thus, Lloyd & Ramshaw (1979) showed that white-brined cheeses manufactured with salt-tolerant strains of lactococci had improved flavour characteristics vis-a-vis control cheeses, and also showed a reduced tendency to develop off-flavours - especially those associated with oxidative rancidity. If this effect is due to organic compounds secreted by the bacteria acting as anti-oxidants, a similar influence might well prove beneficial in Halloumi.

Future Developments

Although standardisation of the age-old process offers one route for increasing production, it has, to date, proved a difficult process to automate. Labbe *et al.* (1985) produced Halloumi successfully from milk concentrated by ultra-filtration, and they noted an increase in cheese yield due to retention of the whey proteins, and a reduction in the time taken for the curd to drain. Obviously this approach could, in theory, increase the output from a factory, but the tedium of the traditional cooking process appears to be the limiting factor. Several attempts have been made to avoid the immersion in boiling whey/brine, and one possibility involves exposing the pressed curd to the action of microwaves (Husain *et al.*, 1986). If the alleged doubts over the organoleptic properties of the finished cheese can be overcome, then the system could handle large volumes of curd, and with a fair degree of automation.

The other popular option would be to manufacture the cheese in regions of high demand, and the use of recombined milks offers an attractive route. Although still under review, it appears to be a feasible option provided that the process conditions are adjusted to reflect the specific characteristics of *the raw materials (Lelievre et al.*, 1990 & 1991). The role of homogenisation

was cited as one factor of special importance, as was the choice of milk powder. The degree of heat treatment received by the skim-milk before drying was found to be critical, and low temperatures improved both the stretch and melt properties of the finished cheese. This problem was also highlighted by Kanafani (1981), who examined the importance of the type of milk powder employed for manufacturing recombined milk for Halloumi. In particular, the latter worker noted that, with medium heat powder, acceptable textural properties could only be obtained with a 50:50 mixture of fresh milk and recombined milk; above the 50% inclusion rate, coherence of the curd declined to an unacceptable degree.

Obviously, Halloumi cheese is not an easy variety to manufacture on a large-scale but, if market demand does increase in the Middle East or elsewhere, one or other of the recent innovations could become a commercial reality.

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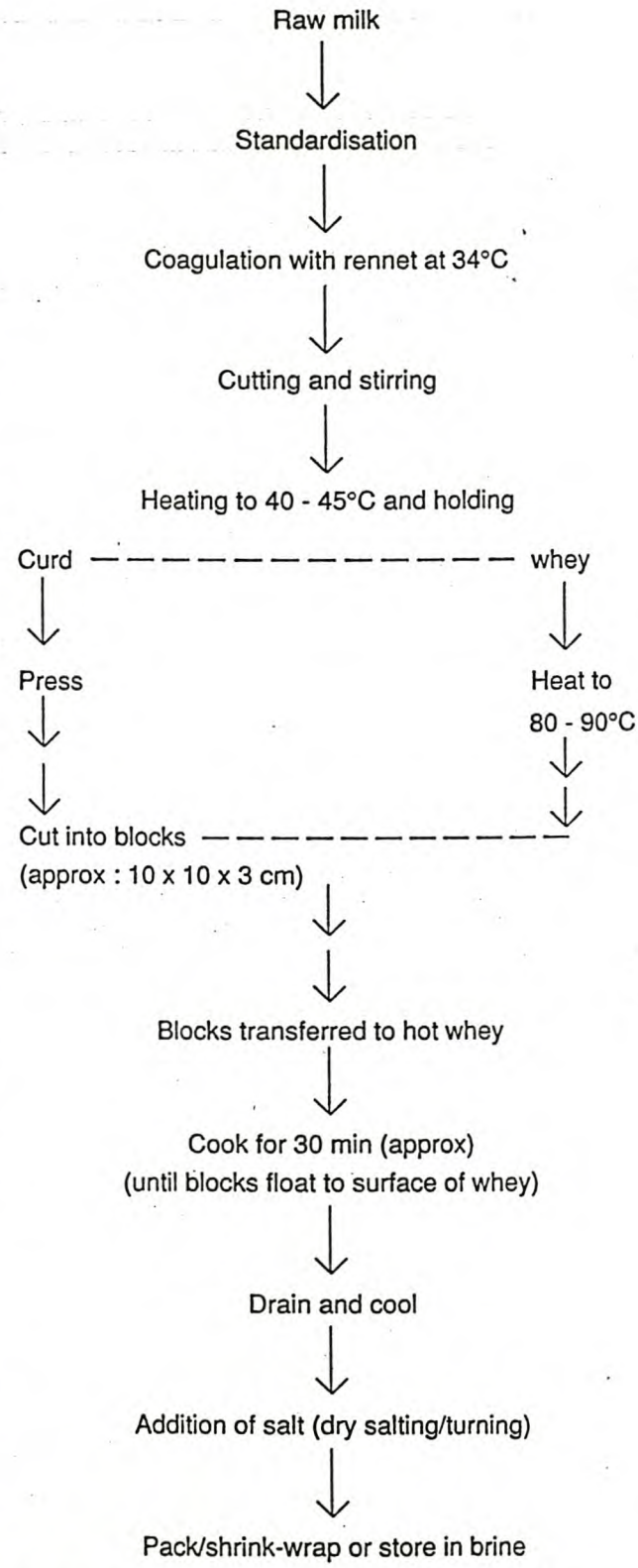

Figure 1 - Flow diagram showing the principal stages in the manufacture of Halloumi cheese

TABLE I

Chemical composition of Halloumi cheese made from sheeps' and cow's milk and of commercial Halloumi from the Cyprus Market (Means of 13, 15 and 17 replicates respectively - all figures *per* 100 g)

Components	Halloumi from sheeps' milk		Halloumi from cow's milk		Halloumi from the Cyprus market	
	Mean	Ranges	Mean	Ranges	Mean	Ranges
Moisture	42.2	39.0 - 43.8	42.9	41.5 - 43.8	42.5	35.5 - 48.6
Fat	27.9	26.3 - 29.3	27.6	26.3 - 28.6	25.6	20.0 - 29.5
Fat in dry matter	48.1	46.1 - 50.0	48.3	47.3 - 49.9	44.5	37.9 - 50.5
Protein (Total N% x 6.38)	23.7	22.0 - 25.0	23.4	22.7 - 24.1	24.5	20.9 - 30.5
Protein in dry matter	41.0	39.2 - 44.3	41.0	39.4 - 42.5	42.5	40.1 - 48.6
Soluble protein (Soluble N% x 6.38)	0.8	0.6 - 0.9	1.1	0.9 - 1.3	1.2	0.8 - 1.6
NaCl	1.4	1.1 - 2.1	1.6	1.1 - 2.2	3.5	2.3 - 5.7
pH	5.9	5.3 - 6.1	6.3	na	na*	na

After: Anifantakis and Kaminarides (1982, 1983)
* data not available

TABLE II

Changes in the total microflora of Halloumi cheese kept in brine (10%) at two different temperures of storage; all counts as colony forming units/g.

	Storage Temperatures			
	4°C		20°C	
Time (days)	Surface	Internal	Surface	Internal
1	8×10^2	2×10^3	9×10^2	4×10^3
4	3×10^3	2×10^3	1×10^7	2×10^6
15	4×10^5	1×10^3	6×10^8	2×10^8
40	7×10^7	4×10^6	4×10^7	6×10^8
60	2×10^8	2×10^7	1×10^7	1×10^7

After: Anifantakis & Kaminarides (1985)

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Halloumi cheese: the product and its characteristics

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Halloumi, the traditional cheese of Cyprus, is extremely popular in the Middle East and the Mediterranean regions of Europe and in recent years exports from Cyprus have risen. The basic cheesemaking process places halloumi in the family of white brined cheeses, but some of its characteristics are quite unique. This paper reviews the studies that have been carried out on the chemical composition, manufacturing procedure(s), sensory quality and storage of halloumi, and considers some proposed developments, such as the use of reconstituted milk powders or homogenized milk.

INTRODUCTION

Cheesemaking is a practice that has been performed for several thousands of years, primarily as a means of preserving the milk. Early records suggest that cheesemaking dates back as far as 6000–7000 BC[1] and, later, Homer *circa* 1184 BC in the classic work *The Odyssey* refers to cheese being made from ovine and caprine milks by the 'Cyclops' in local caves. Herodotus, the father of History, and the great philosopher Aristotle also refer to special cheeses of local origin.[1]

Many of the varieties of cheese that are made today in the eastern Mediterranean and the Middle East are probably derived from these early products, and halloumi may well be among them. Although halloumi was originally only popular in Cyprus, its appeal has spread worldwide, and exports into the European Union (EU), for example, have risen from 300 metric tonnes in 1990 to 764 metric tonnes in 1996; the UK absorbs more than 60% of the total imports into the EU from Cyprus every year.[2]

MANUFACTURE OF HALLOUMI CHEESE

Traditional halloumi cheese, which is of Cypriot origin, is semihard to hard, elastic, has no obvious skin/rind and the texture is close with no holes and it is easily sliced. Its colour varies from white (when ovine or caprine milk is used) to yellowish (when bovine milk is the main ingredient).[3] It can be consumed raw, but it is usually grilled, fried or grated over a hot dish. When halloumi is heated, the texture is comparable to that of the raw product, but the stretch and melt characteristics are altered quite markedly. On heating, molten halloumi has been described as a concentrated viscoelastic polymer solution,[4] in that at low values of applied strain, viscous flow is apparent and elasticity is not so evident, while with increasing values of strain, the response is more elastic and much less viscous. For local use, the cheese may be stored in salted whey for a considerable period of time, and the amount of salt in the whey is important with respect to the stability of the cheese.

As halloumi is a traditional cheese, the procedures of manufacture vary from place to place throughout Cyprus, and this situation creates a lot of confusion as to which is the 'correct' procedure. This issue is a point of some importance, as the Government of Cyprus wishes to protect the identity of the cheese by imposing a uniform system of production.

Specified method of manufacture

The manufacture of halloumi cheese has been assessed during a number of studies,[5–8] and is now controlled by regulations from Government bodies.[9] The overall procedure is shown in Fig. 1, and a crucial step in the manufacturing process is the cooking stage; by law,[9] the blocks of cheese must be heated for at least 30 minutes at a temperature higher than 90°C. After the cooking stage, halloumi cheese attains the 'chicken breast' characteristic of developing cheddar cheese. The blocks (10 × 15 × 3 cm) are then dry salted and sprinkled with crushed leaves of dry mint, *Mentha viridis*, before being folded in half. The blocks are then piled into a convenient container and, after chilling overnight, salted whey is poured into the container until the blocks are covered. This fresh halloumi is kept in the salted whey for approximately 3 hours, before being shrink wrapped ready for distribution. For local consumption, halloumi cheese may be sold in plastic or glass containers filled with salted whey.

Various factors can affect the quality of the finished halloumi, and the time/temperature profile within the cheese during the cooking stage is crucial. Half cooked curds will give an irregular colour (greenish) in the centre of the block, and the rate of temperature

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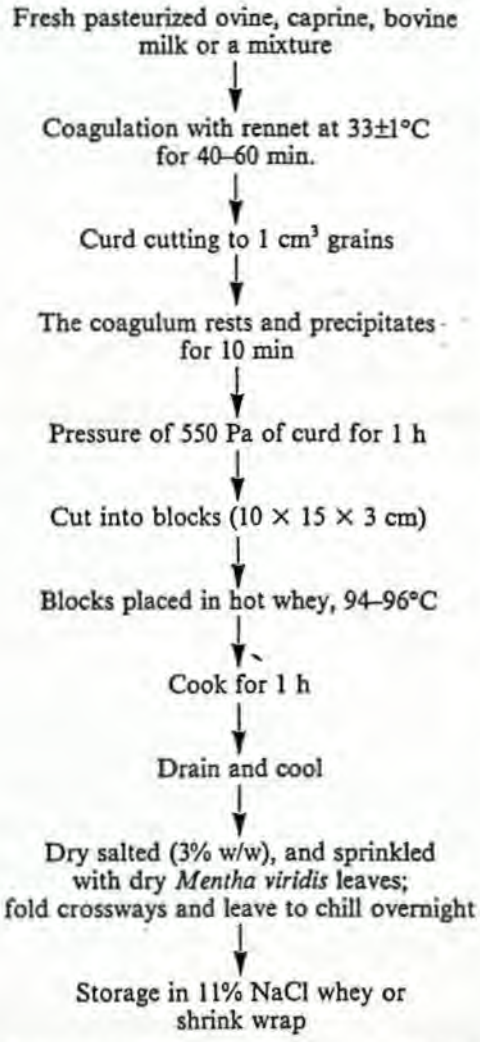

Fig. 1. Traditional process for production of halloumi cheese.

increase in the centre of the block depends heavily on its dimensions.[5] Contrasts between the use of ovine or bovine milks have been examined as well,[6] and differences in the percentages of fat and protein between the two milks affected the yield of the cheese. Alterations in the technology were rated as important in the following respects: (i) the cooking time of the cheese should be shorter when using ovine milk alone; and (ii) the pressure applied to the initial curd in order to expel the whey has to be greater and applied for a longer period of time when bovine milk is the main ingredient. Economides *et al*[10] employed pure ovine, caprine and bovine milks for the production of halloumi cheese, and a mixture of equal parts of ovine and caprine milks. The chemical composition of the cheeses was determined (see Table 1), and multiple linear regressions were utilized to develop prediction equations for cheese output from the four types of milk. Total fat, protein and casein were regarded as important variables in predicting cheese output, as was the casein to fat ratio.

Chemical composition

According to the Cypriot Standards, the finished product should contain a maximum 3% of sodium chloride and a minimum fat-in-dry matter level of 43%. The maximum moisture content for halloumi cheese is 46%. Most products on the market meet these requirements, and some typical analyses are shown in Table 1.

Shaker *et al*[8] examined which manufacturing variables influenced the chemical composition and physical and sensory properties of halloumi cheese made from bovine milk. The composition was measured in terms of moisture-non-fat substance (MNFS), fat-in-dry matter (FDM), salt-in-moisture (S/M) and pH. The calcium to solids-non-fat-non-salt (Ca/SNFNS) was also determined as it could be an important factor with respect to the stretchability of heated cheese. The authors

TABLE 1

Chemical composition of halloumi cheese made from ovine, bovine, caprine and mixed (equal parts of ovine and caprine) milk and of commercial halloumi cheese from the Cyprus market

	Traditional halloumi (sheep's milk only)		*Halloumi from cows' milk*		*Halloumi from goats' milk*		*Halloumi from mixed milk (equal parts of sheep and goat milk)*		*Halloumi from the Cyprus market*	
Components	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Moisture	42.2	39.0–43.8	42.9	41.5–43.8	43.5	na	42.5	na	42.5	35.5–48.6
Fat	27.9	26.3–29.3	27.6	26.3–28.6	23.7	na	25.8	na	25.6	20.0–29.5
Fat in dry matter	48.1	46.1–50.0	48.3	47.3–49.9	41.9	na	44.9	na	44.5	37.9–50.5
Protein (total N% × 6.38)	23.7	22.0–25.0	23.4	22.7–24.1	24.8	na	23.6	na	24.5	20.9–30.5
Soluble protein (soluble N% × 6.38)	0.8	0.6–0.9	1.1	0.9–1.3	na	na	na	na	1.2	0.8–1.6
NaCl	1.4	1.1–2.1	1.6	1.1–2.2	2.2	na	2.0	na	3.5	2.3–5.7
pH	5.9	5.3–6.1	6.3	na	na	na	na	na	na	na

After: Anifantakis and Kaminarides[6,7] and Economides *et al*.[10]
na = not available.

concluded that improved stretch and melt characteristics, and a tender mouth feel of the heated cheese, could be achieved by lowering both the pH of the cheese and the Ca/SNFNS ratio. Tenderness with respect to mouth feel also improved at high levels of MNFS.

Although the traditional cheese is always made with ovine milk—a raw material that is in short supply, the growing market demand for halloumi has raised the fear that manufacturers may attempt to adulterate ovine milk with the bovine equivalent. The possibility of detecting bovine milk in halloumi cheese that should be of ovine origin was examined by Kaminarides *et al*[11] using polyacrylamide gel electrophoresis (PAGE). This approach was based on the fact that the electrophoretic mobility of bovine and ovine α_{s1}-caseins differ, with bovine α_{s1}-casein showing more rapid migration. The electrophoretic profile of this specific casein (α_{s1}) was chosen because it remains almost intact during both cheese manufacture (cooking of the curd) and cold storage under brine (7% NaCl).

For the detection of bovine milk, samples of halloumi cheese at 1 day and 40 days after production were subjected to PAGE. A densitometer, adjusted with the α_{s1}-casein band from genuine bovine halloumi cheese, was used to quantify the level of bovine milk present in the adulterated samples by expressing the density of the band for bovine α_{s1}-casein of each sample as a percentage of that from genuine bovine milk. The authors detected percentages as low as 2.5% bovine milk in samples of ovine halloumi cheese, irrespective of the age of the cheese. Linear relationships between the densities of the bovine α_{s1}-casein bands in the adulterated samples and the percentages of bovine milk in the cheeses were found to be between 2.5% and 10% levels of adulteration, and regression equations were produced. These equations made it easy to calculate levels of bovine milk higher than 2.5% in cheese samples of unknown composition.

Although Tamime *et al*[12] suggested that immunological techniques could be employed to detect the adulteration of ovine milk with bovine milk used for the manufacture of kishk, Kaminarides *et al*[11] believed that the severe heat treatment employed during the manufacture of halloumi cheese would, in the present context, invalidate the procedure.

TABLE 2
Some physical properties of halloumi cheese, along with some comparable information on mozzarella (see text for further details)

Source of milk	Stretchability (cm)	Meltability (% increase in area)
Halloumi cheese		
Fresh bovine milk	25	342
Recombined milk (SMP + AMF) homogenized at 0.4 MPa	–	276
Recombined milk (SMP + AMF) homogenized at 6.7 MPa	7	133
Mozzarella cheese		
Fresh bovine milk	53	ND
Recombined milk (SMP + AMF) homogenized at 0.4 MPa	15	230
Recombined milk (SMP + AMF) homogenized at 6.7 MPa	9	193

After: Shaker *et al*[6] and Tamime *et al*.[12]
ND = not determined.

Physical properties of halloumi

As mentioned above, halloumi cheese should melt and stretch on heating, which means that the compact texture of the cheese will be partly lost and a degree of flow introduced to the melted cheese. These properties are often referred to as the 'meltability' and 'stretchability' of the cheese and, although obvious to the consumer, they are characteristics that are not easy to quantify.

Nevertheless, attempts to assess these features and, in one study, the meltability of halloumi cheese was determined[8] by measuring the percentage increase in area after cubes of cheese (2 cm sides) were placed in sealed aluminium dishes and held in a boiling water bath for 25 minutes. The stretchability was measured by placing pieces of cheese prewarmed in a water bath at 85°C for 4.5 minutes onto the base plate of an Instron Testing Instrument (Instron Ltd, High Wycombe, UK), and then with a special hook, stretching the soft cheese until the strand broke; the length of the strand at the point of breaking was recorded. The relevant figures are shown in Table 2, along with some comparable data relating to mozzarella cheese.[4,13,14] An alternative method for measuring meltability is the Schreiber Test,[15] which involves positioning round samples of cheese in glass petri dishes, and then placing the dishes in an oven at 232°C for exactly 5 minutes.[16] The dishes are removed from the oven, and the increase in diameter of the melted cheese over the original is recorded after approximately 30 minutes.

However, the problem with tests of this type is that they are rarely reproducible, and the comparison of the results in Table 2 for meltability suggests that the variation is between techniques rather than products; it is most unlikely that halloumi cheese would spread on heating more than mozzarella. Nevertheless, the values for relative stretchability are more in line with the expected results, in that the properties of mozzarella made from fresh milk are quite different from those made from the recombined material; the same appears to be true for halloumi.

The effect of homogenization pressure on the physical properties of mozzarella cheese can be seen from the results obtained by Lelievre *et al*.[4] The most likely explanation is that, as the homogenization pressure is raised, the increased number of small fat globules retain additional casein at the fat–water interface. The formation of permanent crosslinks between the casein micelles then effectively traps the fat phase, so reducing the flow properties of the

heated cheese; a similar trend is evident with halloumi cheese. This hypothesis also explains the action of lecithin, in that by forming a surface film over the fat globules, the lecithin coating prevents the casein micelles from adsorbing onto the fat–water interface; the crosslinking reactions are, therefore, limited, so leaving the cheese to stretch and flow under stress.

Whether or not the use of recombined milk has exaggerated the proposed interactions is not clear, but a similar effect was reported by Apostolopoulos,[13] who compared the stretchability of mozzarella cheese manufactured by a standard procedure with the properties of mozzarella cheese made with homogenized milk (17.2 MPa). In the case of the standard cheese, the average stretchability was 53 cm but, probably for the reasons given earlier, homogenization reduced this figure to 27 cm.

Neither the ripening time during manufacture nor time in storage affect significantly the stretch values of mozzarella cheese,[13] probably because any potential proteolytic activity from residual coagulant is prevented by the heat treatment of the curd (58–60°C). This inactivation means that any degradation of the paracasein is limited as, consequently, are changes in the melting characteristics of the cheese. The cooking of halloumi is likely to have a similar effect, but the influence of other aspects of processing might merit further attention.

For example, Renda *et al*[16] reported that the meltability of mozzarella cheese was affected by the speed of mechanical mixer screws used for stretching the curd during manufacture. In particular, the highest screw speed resulted in a cheese with lower moisture and FDM contents, and these compositional changes reduced the meltability values obtained with the Schreiber Test. Processing variables, such as the time and temperature of pressing, that could affect the moisture content of halloumi cheese could be equally important, and this aspect of halloumi manufacture could merit further attention.

Sensory features of halloumi

The analysis of volatiles of halloumi and their relationship with flavour has been reported,[17] and a panel of 11 assessors used Quantitative Descriptive Analysis to profile the flavour and texture attributes of raw, fried and grilled halloumi cheese. Principal Component Analysis of the sensory data highlighted significant differences between individual product–cooking combinations, and specific analyses of the flavour components differentiated between sweet and sour sensations. The descriptors for texture ranged from coarse and grainy to milky and creamy, and the 'milky, creamy and fatty' characters of halloumi cheese were easily detected by assessors when the cheese was heated.

The volatiles in halloumi cheese, as detected by gas chromatography (headspace analysis) have little in common with other brined cheeses like feta or domiati, as the cooking of the curd causes considerable losses. Indeed, the main volatiles found in commercial halloumi cheese were an unidentified lactone, probably arising from the breakdown of fat, and some alcohols, acids and phenols arising from the degradation of amino acids. Partial Least Square regression analysis was used to predict mean sensory scores from the headspace data, and a 'minty' flavour was predicted successfully from the presence of pulegone, 'mint terpene' and carvone. The presence of a fatty flavour was loosely predicted on the basis of two compounds only—acetic acid and an unidentified compound—while predictions of creamy and milky associated with nonanone and phenol were adequate.

Two points worth mentioning in this context are: (i) the complete absence of starter cultures and/or enzymes in both the traditional and current cheesemaking practices apart, of course, from the rennet which is used to coagulate the milk and the natural enzymes present in the milk; and (ii) traditionally, ovine and/or caprine milks were used for the manufacture of halloumi but nowadays the large dairies use bovine milk almost exclusively. This gradual transition towards bovine milk has resulted from the low levels of production of ovine and caprine milks, but the change has had an impact on the sensory quality of the cheese, since the 'old' aroma and flavour have been lost.

The use of starter cultures to ripen pasteurized milk prior to coagulation has been suggested as a means to enhance the flavour profile,[18] and a mixture of mesophilic bacteria, eg, *Lactococcus lactis* subsp *lactis* and *Lactococcus lactis* subsp *cremoris*, or thermophilic species like *Streptococcus thermophilus* might be considered. Papademas (unpublished data) has examined the employment of coccus: rod mixtures of thermophilic microorganisms, such as *Strep thermophilus* and *Lactobacillus helveticus*, a combination that shows both high proteolytic and acidifying activity.[19–21] Whether this proposal will provide halloumi from bovine milk with a more intense flavour remains to be established.

Storage conditions and microbiology

It has been determined that halloumi cheese can be stored successfully for a week at 20°C or for 40 days at 4°C and during storage the salt and moisture contents increase as the cheese absorbed brine.[22] The change in levels of soluble nitrogen was minimal during storage, so confirming that proteolytic activity is limited. This latter observation is accounted for by the high temperatures employed when the curd is cooked, which eliminates most of the microorganisms that could initiate proteolytic and/or lipolytic action; $>10^3$ colony forming units (cfu) g^{-1} were reported for fresh halloumi.[22]

TABLE 3
Changes in the total colony count and yeast counts in halloumi cheese kept in brine (10%) at two different temperatures of storage; all counts as colony forming units/g

	Total colony count				Yeasts			
	4°C		20°C		4°C		20°C	
Time (days)	Surface	Internal	Surface	Internal	Surface	Internal	Surface	Internal
1	8×10^2	2×10^3	9×10^2	4×10^2	Nil	Nil	20	Nil
4	3×10^3	2×10^3	1×10^7	2×10^6	10	Nil	20	5
15	4×10^5	1×10^3	6×10^8	2×10^8	10	Nil	1×10^5	9×10^2
40	7×10^7	4×10^6	4×10^7	6×10^9	60	Nil	5×10^5	4×10^2
60	2×10^8	2×10^7	1×10^7	1×10^7	5×10^3	Nil	2×10^4	1×10^3

After: Kaminarides and Anifantakis.[24]

However, as shown in Table 3, halloumi cheese is by no means free from bacteria and after only 4 days' storage at 20°C the number of microorganisms in the centre of a block of cheese increased to $>10^6$ cfu g^{-1}; during cold storage, it took around six weeks to reach the same figure. Spore formers like *Bacillus* contribute to these microflora, but thermoduric species in the milk and contaminants from the salt or mint leaves will elevate the numbers as well. Yeasts are often isolated from halloumi cheese, particularly on the surface of the individual blocks, and the presence of postpasteurization contaminants raises the question of safety. For example, Gohil *et al*[23] found that *Listeria monocytogenes* could survive in the brine used to transport feta cheese and, although the numbers were probably low, the result did highlight the need for high standards of hygiene operating during cheese packaging.

The option of employing high salt concentrations to eradicate pathogens has been considered, but while *L monocytogenes* may be inhibited by sodium chloride concentrations above 10%, Kaminarides and Anifantakis[24] showed that around such levels flavour becomes the major consideration. In addition, the Regulations in Cyprus state that the maximum salt content in the retail cheese should be 3%.

ALTERNATIVE PROCEDURES FOR MANUFACTURE

Use of milk powders in the production of halloumi cheese

Since halloumi has become very popular in the Middle East and fresh milk is not available in any quantity, the use of reconstituted skim milk powder (SMP) blended with anhydrous milk fat (AMF) has been considered. Some of the preliminary results have been discussed, but modification of the properties of the milk powder could offer a route for improvement.

In particular, Lelievre *et al*[25] modified skim milk powder by changing its preheat treatment from 68 to 72°C, and examined powders that were manufactured at different times throughout the year, ie, spring, summer and autumn. Additional batches of halloumi cheese were made from process milks in which high protein powders—derived from ultrafiltered (UF) milk—were reconstituted to give 16.8% protein; the use of UF skim milk powders from milks fortified with calcium chloride or acidified was explored as well.

The results showed that the degree of the preheat treatment of the SMP powders had a statistically significant effect on the physical properties of the final cheeses, but not on their chemical composition. In particular, low heat treated powders performed better than the high heat ones in producing a cheese with the stretching and melting properties expected of halloumi cheese, and O'Keefe and Phelan[26] arrived at the same conclusion. As far as the performance of the high protein powders was concerned, the stretch and melt characteristics were reasonable for cheeses made from both normal (3.5% protein) and concentrated (16.8% protein) recombined milks.

The differences in composition between the autumn and summer powders was important, and the better values for stretchability and meltability were observed in halloumi cheese made from skim milk powder manufactured in the autumn. The physical properties of halloumi cheese were also affected by the various calcium levels in the UF powders. Thus the stretching and melting properties were more pronounced in cheeses made from milks based on UF powders derived from acidified milk than in the control cheeses or those made from calcium fortified powders. The low level of calcium in the acidified milk powder was alleged to be responsible for the better melt and flow characteristics, since the formation of permanent calcium phosphate crosslinkages, which prevent the cheese from flowing on heating, was reduced. Overall, the results seemed to demonstrate that halloumi cheese with good physical properties can be produced from recombined milks, as long as the calcium/SNFNS ratio is low.

Process and quality control

Robinson *et al*[3] and Robinson[18] reviewed a number of studies in which particular attention was paid to increasing the output and quality of halloumi cheese. The use of fresh milks concentrated by ultrafiltration was considered to offer some advantages with respect to plant requirements as, due to the retention of the whey proteins, this approach would increase the yield of the cheese; however, the impact on quality would need careful consideration. The suggested exposure of the pressed curd to microwaves[3] as an alternative to the traditional cooking stage has been proposed as a system that would allow for an increase in automation; the effect of a microwave treatment on the sensory properties of the finished cheese does, however, need further investigation. In addition, as the procedure is not mentioned in the Cyprus Standards for the manufacture of halloumi

cheese, the use of the procedure could cause legal problems as far as the identity of the cheese is concerned.

Some standard methods for the sampling of milk and milk products are included in the Cyprus Standards, as are methods for the chemical and microbiological analyses of the same items.[27–29] The compilation of such standards has proved to be valuable as far as the drive to improve the quality of halloumi cheese is concerned, but finding uniform methods of analysis for the physical or organoleptic properties of the product may be more difficult.

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Properties of full-fat, less-fat and reduced-fat halloumi cheeses made from bovine milk

By Photis Papademas, Julia M. Norman and Richard K. Robinson, Department of Food Science and Technology, The University of Reading, Britain.

Halloumi cheese is the traditional cheese of Cyprus and, over hundreds of years, it has been manufactured from raw ovine or caprine milks, alone or as blends.

However, in recent years, the manufacturing capacity for halloumi has expanded rapidly to meet the growing demand in the Middle East and Europe, and the regulations in Cyprus now allow the more readily available bovine milk to be used in place of the traditional ovine or caprine milks.[1,2]

This transition has been broadly successful and, while the manufacturing procedure for making halloumi from pasteurised bovine milk has some features different from the traditional process, studies have found the end-product to be comparable, in most respects, with halloumi of ovine or caprine origin.[3,4,5,6,7]

Indeed, differences between the cheeses only become apparent if a good-quality, fresh ovine product is compared directly with a similar cheese made from bovine milk, and even this difference tends to fade as the two types of cheese mature.

A further pressure on the dairy industry in Cyprus to manufacture halloumi from bovine milk arises from the flourishing tourist industry, for supplies of ovine or caprine cheeses are just not sufficient to cope with the summer demand.

In addition, bovine halloumi has two specific attractions for tourists:

- the retail price is lower than that of the ovine/caprine product; and
- it could, perhaps, be manufactured as a low-fat variant.

Thus, many visitors from Northern Europe and North America have been persuaded to buy cheeses with fat contents lower than their traditional counterparts on the grounds that high intakes of saturated fats should be avoided. As a consequence, shops are now expected to display low-fat varieties of popular cheeses such as cheddar, gouda and mozzarella that have been developed for this niche market,[8,9,10,11,12,13] and even the potential of low-fat feta cheese has been examined.[14]

Despite problems with taste and texture limiting the market share for low-fat cheeses,[15,16,17] Bruhn *et al.*[18] found that many 'health-conscious' consumers in Europe would be willing to pay more for low-fat products than for the comparable full-fat ones, and would certainly buy them if the organoleptic properties matched those of the traditional food.

If some of these ideas are valid, then the overall conclusion would appear to be that dairies in Cyprus and, perhaps elsewhere, should seek to exploit the demand for low-fat Halloumi made from bovine milk and, at the same time, benefit from the additional income derived from the sale of the separated cream.

However, if the manufacture of low-fat halloumi is to be expanded, then its properties must be comparable with those of the normal cheese. Hence, the aim of this project was to:

- produce less-fat and reduced-fat samples of halloumi in the laboratory; and
- compare their properties with a normal, full-fat cheese (control) made under identical conditions.

Figure 1: The process used to make three types of Halloumi cheese.

Pasteurised bovine milk with fat content adjusted
/
Coagulation with rennet at 32/33°C for 40-60 min
/
Cutting of the curd (1cm³ cubes)
/
Precipitation and drainage of curd with pressure of ~500Pa for 1h
/
Cut into blocks (10 x 15 x 3cm)
/
Place blocks in hot whey (~95°C) for 30-60 min., then drain and cool
/
Dry salt (30g/kg), sprinkle with dry leaves of mint (*Mentha viridis*), fold crossways and chill overnight
/
Mature in brine (~11% NaCl) and shrink wrap for testing as fresh Halloumi

Manufacture

Although the nomenclature for low-fat products varies from country to country, some markets have recognised two cheese categories: 'less-fat cheese' containing at least 25% less fat than normal cheese of the same variety, and 'reduced-fat cheese' with at least 50% less fat than normal.[19]

In the case of halloumi, with an analysis of about 25.75±3.14% fat,[7,20] the comparable specifications would be <20% fat for a less-fat cheese and <15.0% fat in a reduced-fat brand.

Pasteurised, full-cream milk (3.5% fat and 3.3% protein) was used to make the control cheese, while semi-skimmed and skimmed milks formed the bases for the less-fat and reduced-fat cheeses, respectively.

For the less-fat cheese, the fat content of the semi-skimmed milk was adjusted from 1.5% to 2.3% with cream (18% fat), while the fat content of the skimmed milk was adjusted to 1.3% from a base of 0.5% fat for making the reduced-fat cheese.

The manufacture of halloumi in Cyprus is controlled by government regulations.[2] These guidelines were followed to produce the experimental cheeses in the laboratory. The overall procedure is shown in Figure 1 and, even though the milk was pasteurised, a starter culture was not needed.

Each batch of cheese was made from 12L of milk and, after coagulation with rennet, a layer of curd (2-3cm in

depth) was spread over a perforated stainless steel tray (40 x 30cm) lined with cheese cloth. The cheese cloth was then folded over the curd and a pressure of ~500Pa applied by means of a stainless steel plate (39 x 29cm).

After an hour, the cheese was firm enough to cut into blocks (10 x 15 x 3cm), and these were then transferred to a vat containing the whey that had drained from the original coagulum.

The blocks were then 'cooked' for 30-60 minutes at 90-95°C. By that time, the blocks had floated to the surface of the whey. They were then removed and cooled to room temperature.

The exposed surfaces of each block were sprinkled with salt and crushed leaves of dry mint, *Mentha viridis*, before being folded in half.

After chilling overnight in a convenient container, salted whey was poured into the container until the blocks were covered. Following a holding period of about three hours in the salted whey, the halloumi was shrink-wrapped for storage at 7-8°C.

The entire procedure was completed on two occasions for each of the three types of cheese – full-fat, less-fat and reduced-fat.

For the sensory analysis, only one of the two available batches of cheese was examined.

Properties of the cheeses

Chemical composition

According to the Cyprus Standards,[2] normal halloumi should contain a maximum of 3% sodium chloride, have a minimum fat-in-dry-matter content of 43%, and a maximum moisture content of 46%.

Table 1: Chemical composition of the three types of Halloumi cheese; all figures as g/100g of cheese as consumed, except (%) fat-in-dry-matter (FDM); all analyses were completed in accordance with the methods described by Kirk and Sawyer.[26]

	Full fat	Less fat	Reduced fat	Published data[a]
Moisture	45.2± 0.02	52.7± 0.08	54.7± 0.29	42.9
Fat	28.0± 0.0	19.3± 0.35	10.8± 0.35	27.6
FDM	51.0	40.8	23.8	48.3
Protein	21.1± 0.57	24.4± 1.1	27.9± 1.1	23.4
Salt	3.6±0.42	2.5± 0.03	3.7± 0.05	1.6

[a] *Mean composition of Halloumi cheese made from cow's milk.*[3]

However, these figures are for top-quality halloumi made from ovine/caprine milk and it is likely that cheese made from bovine milk will differ slightly from these standards.[3]

The analyses of the full-fat, less-fat and reduced-fat halloumi are shown in Table 1, along with some published data for typical bovine halloumi from the market.

Although the salt content exceeds the legal limit, it is evident that the full-fat cheese is broadly similar to the commercial brands. Equally noticeable is the fact that, as the fat contents were reduced, so the levels of moisture and protein increased.

From a nutritional standpoint, it could be argued, therefore, that the low-fat cheeses do offer a healthy option but, as highlighted earlier, this is only useful if the sensory quality of the food is also acceptable.

Physical properties

Some of the basic physical properties of the cheeses as measured by the Texture Profile Analyser,[21] e.g. springiness or cohesiveness, were similar for all cheeses, but both fracturability and hardness were significantly lower in the full-fat cheese (see Figure 2).

This difference between the full-fat and the less/reduced-fat cheeses is probably a reflection of the higher protein contents rather than the changes in fat or moisture, for there is a well-established link between protein content and the hardness of cheese.[14,22]

A small, but significant, difference in chewiness between the full-fat and the other two cheeses was also noted and, again, the increase in protein was probably responsible.

As halloumi is often eaten grilled or fried, it was assumed that the test samples should, at least to some degree, melt and soften on heating, so that the compact texture of the cheese would be partly lost and an element of 'flow' introduced into the cheese structure.

The resultant properties are often referred to as the 'meltability' and 'stretchability' of the cheese and, although obvious to the consumer, they are characteristics that are not easy to quantify.[7,23,24]

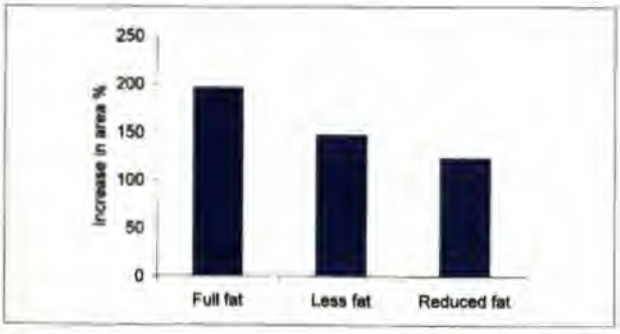

Figure 2: A comparison of some textural properties, as measured with the Texture Profile Analyser, of three samples of Halloumi cheese with different fat contents.

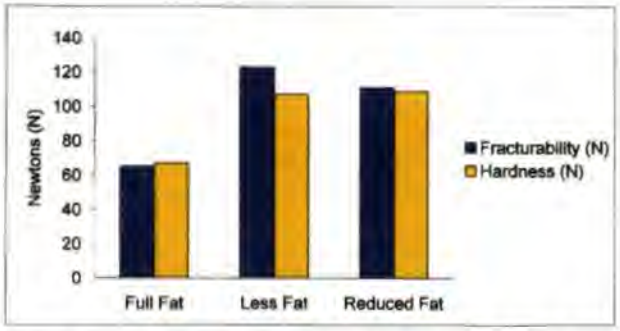

Figure 3: Melting properties of Halloumi cheese with different fat contents. Figure shows increase in area of heated sample compared with area occupied by sample before heating.

In this study, the meltability of the halloumi was determined by measuring the percentage increase in area occupied by a cube of cheese (2 x 2 x 1cm) following exposure in a microwave oven (800 watt) for five minutes.

Stretchability was measured by placing a piece of cheese (1 x 1 x 0.5cm) pre-warmed in a water bath at 85°C for 4.5 minutes onto the base plate of an Instron Testing Instrument (Instron Limited, High Wycombe, UK) and then, with a small hook, stretching the soft cheese until the strand broke; the length of the strand at the point of breaking was then recorded.

Each test was completed with 10 cubes/pieces of cheese per batch.

The mean results for meltability are shown in Figure 3, and there was a significant difference between the full-fat cheese and the less/reduced-fat samples.

The lubricating effect of the fat in allowing sub-units of the protein matrix to slide over each other is not unexpected, but it was clear that the fat content had to be >20% to have any significant effect.

A similar trend was noted with respect to stretchability, with the full-fat cheese giving an extension of 9.08±1.5mm compared to 6.71±0.9 and 7.18±1.8 for the less-fat and reduced-fat cheeses, respectively. Again, there was no significant difference between the less-fat and reduced-fat cheeses.

The increased hardness of the fresh cheese with <20% fat may not be a problem (see later) but, even so, it was evident that lowering the fat content of the test cheese below 20% changed the physical characteristics of the cheese quite dramatically; further reductions in fat content had less impact.

Whether the reduced meltability and stretchability of the less/reduced-fat cheeses would limit their appeal for use in pizza toppings or as components of cheese burgers is not clear, but mixing them with other cheese could solve any serious limitations.

Sensory properties

A panel of 11 partially trained assessors used Quantitative Descriptive Analysis to profile the flavour and texture attributes of the three types of halloumi.[25] The results are shown in Table 2.

Table 2: Average sensory scores (maximum 100/attribute) for the three types of Halloumi cheese.

Attribute	Full-fat	Less-fat	Reduced-fat
Colour	58	58	48
Hardness	47	63	83
Crumbliness	48	38	50
Chewiness	48	60	62
Moistness	51	48	36
Saltiness	38	40	54
Creaminess	38	34	25
Hedonic response	46	42	47

As with the instrumental analysis, hardness was the attribute that changed significantly with fat content, although it was notable that increasing hardness did not change the hedonic response of the tasters.

The absence of any negative reaction to the increasing hardness of the lower-fat cheeses could well derive from the fact that the tasters were all from England, and for such consumers, the hard-pressed territorial varieties of the UK tend to provide inherent expectations with respect to firmness/hardness of a cheese.[27]

Similarly, the neutral hedonic response to all three cheeses suggests that the panellists found the organoleptic properties of halloumi different from other cheeses with which they were more familiar.

What could be important, however, is that there was no discrimination against the less/reduced-fat cheeses. It could be concluded that 'occasional' consumers of halloumi would accept low-fat versions without complaint.

This tentative conclusion could be good news for the dairy industry in Cyprus for, even if it is assumed that native Cypriots will retain a preference for the traditional full-fat halloumi made from ovine/caprine milk, a major market for low-fat halloumi could be generated within the hotel, tourist or export markets.

Thus, the equal acceptance by the taste panel of both the full-fat and less/reduced-fat cheeses suggests that health-conscious consumers not familiar with the specific characteristics of traditional halloumi may well be more than happy with the lower fat cheeses – a state of affairs that should please manufacturers and consumers alike. ❐

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CASEIN PROPERTIES IN RECOMBINATION

The influence of raw materials on the structure of a Feta-style cheese made by direct recombination. By M Z Ali and R K Robinson

Figure 1. Above (left): An example of the type of white brined cheese that is popular in the Middle East. Although it does fracture in the conchoidal manner typical of brined cheeses, the overall texture is soft and close compared with Gibna Baida (right).

Gibna Baida is the traditional white-brined cheese of Sudan (1), a firm, open-textured, high salt cheese that is usually consumed along with beans or salads.

In an earlier paper (2), the authors demonstrated that a cheese, similar in organoleptic characteristics to Gibna Baida, could be manufactured by the direct recombination of skim-milk powder, sodium caseinate and anhydrous milk fat. However, it was noted that the test cheese was very different in texture from white-brined cheeses found in other markets which, by contrast, were often softer and more smooth in texture (see Figure 1). It was surmised that this difference could have been due either to the contrast in raw materials, eg fresh milk for the Feta-style cheeses, or to the fact that most traditional cheeses have been subject to pressing, ie are textured cheeses as against 'cast' types.

As the aim of the recombination process is to avoid the use of both fresh milk and expensive plant, it was decided to examine the possibility of modifying the structure of the experimental cheese by changing the type of caseinate employed. A study was instigated to determine:

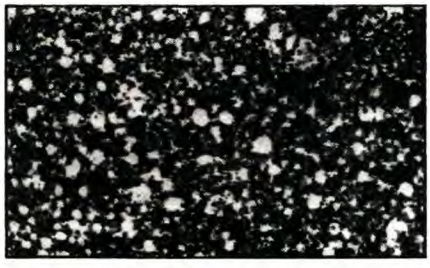
- the properties of those sources of casein that might be suitable for use in the recombination process;
- whether any of the selected sources would influence the gross and/or microscopic structure of the test cheeses.

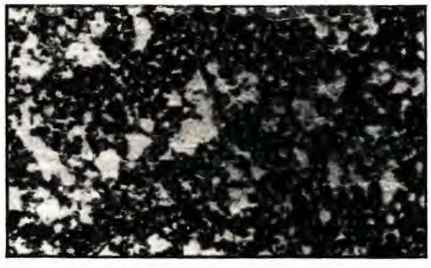
Materials and methods

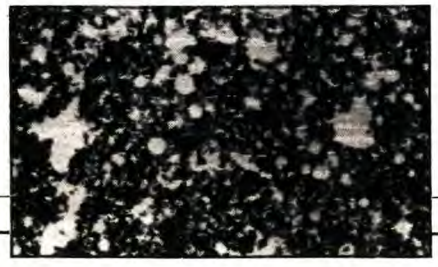
The materials selected as possible bases for the test cheeses were acid and rennet casein, along with the caseinates of sodium, potassium and calcium. Standard commercial samples obtained from Ludwig Post Gmbh & Co, Heidelberg, Germany were examined for:

- moisture content by air drying a sample (2g) at 102°C until a constant weight had been achieved;
- acidity by both pH and titratable acidity; a 10g sample was added to 60ml of distilled water (40°C), and the suspension blended with a stomacher. After standing for 20 minutes, a 10ml sub-sample was filtered through a glass filter prior to titration against N/9 NaOH; the results were recorded as % lactic acid. The supernatant remaining in the original flask was decanted and the pH measured directly with a standard combination electrode;
- solubility of 10% suspensions of the different caseins and caseinates was determined by the method described in (3);
- ash and crude protein by the methods described in (3).

Figure 2. Light micrographs showing the relative porosites — as indicated by the transparent areas — of one day-old test cheeses made with (a) sodium, (b) calcium and (c) potassium caseinates







On basis of these examinations, it was decided to use only the caseinates for cheesemaking, and the direct process of manufacture has been described elsewhere (2). The physical structure of the test cheeses was examined both by light microscopy, to obtain a comparison of coarseness as it might be perceived by a consumer, and by scanning electron microscopy.

For examination (under the light microscope, fine sections (3μm) of each of the test cheeses were cut with a sliding microtome fitted with a Frigister stage cooler and knife-freezing attachment. As the sections left the knife, they were placed directly onto a glass slide and allowed to air-dry. The sections were then fixed in 10% formaldehyde (95% ethanol solution) for 20 minutes, washed in tap water for 2 minutes and stained in carbol fuchsin/phenol solution (basic fuchsin 1.0g; absolute alcohol 10ml; 5% phenol in distilled water 100ml) diluted with an equal volume of distilled water for 10 minutes. The sections were then rinsed with tap water (2 minutes), before dehydration through an alcohol series and xylene ready for sealing with DPX mountant.

Selected sections were placed on a microscope stage, and a number of fields chosen, at random, for photographing with Ilford Pan F film. The total magnification at the film was X 160, and each negative was enlarged positively X 2 onto a high-contrast transparent film.

The apparent coarseness of any given sample of cheese was calculated as follows (M L Green, personal communication). The transparent photograph (46mm x 69mm) was placed onto a perspex sheet of the same dimensions (situated over a light box), onto which had been drawn a series

of equally-spaced black lines — 18 lines cross-ways and 29 lines along the long axis. The total length of these lines, ie 46mm x 18 and 69mm x 29 equalled 2,829mm. A count was then made of the total number of times that a segment of line was visible through the section of cheese, and comparative coarseness was calculated as:

$$\text{Coarseness} = \frac{1/\text{total number of visible segments of line}}{2{,}829}$$

Four sections of each test cheese were examined in this way, and the results provided an arbitrary measure of the texture of the sample. Samples were studied under the electron microscope using the procedure described in (2).

Results and discussion

The results of the chemical analyses are shown in Table 1, and it is clear that neither of the caseins have the degree of solubility required for easy use in the present system of cheesemaking; the low pH of the acid casein was undesirable.

Table 1. Some physico-chemical analyses of the caseins and caseinates considered for the manufacture of Feta-style cheeses by direct recombination; all figures as (%), and each reading is the average of three samples.

Material	Moisture	pH	Acidity (as 1.a)	Solubility	Crude* Protein	Ash
Acid casein	8.61	4.95	0.05	45	87.3	4.34
Rennet casein	6.51	6.90	0.06	57	88.2	4.19
Calcium caseinate	5.80	6.55	0.21	75	91.5	4.51
Potassium caseinate	6.09	6.85	0.21	98	90.9	4.46
Sodium caseinate	6.12	6.95	0.21	99	91.2	4.42

** Total nitrogen X 6.38*

Test cheeses were, therefore, only manufactured employing the three types of caseinate, and a comparison of coarseness as assessed by the light microscope is shown in Table 2, together with the result for a market sample of Gibna Baida. What the data shows that on the first day after manufacture, the sodium caseinate cheese had a more compact structure than those derived from the other caseinates, and this conclusion is borne out by Figure 2. Thus it is apparent from the photographs that the whey-filled spaces in the cheeses made from calcium caseinate (b) and potassium caseinate (c) are generally much larger than in the sodium caseinate cheese, and hence that the former cheeses have a more coarse structure.

As the cheeses matured, so the numerical values converged, and it would appear from Table 2 that all the cheeses were broadly similar in texture by the end of one month. This conclusion was substantially true as revealed by this particular technique, for the sodium caseinate cheese did develop larger whey filled apertures and under the light microscope it appeared not unlike the potassium caseinate cheese (c) in Figure 2.

However, this apparent similarity did not, in the event, truly reflect what was happening during the maturation process. A physical examination of the cheeses at one month showed that the sodium caseinate sample was like the traditional Gibna Baida, while the other samples were semi-soft and smooth in texture. Clearly, therefore, the microscopic technique for measuring coarseness could not cope with materials of such complex structure, but whether it could be applied to other materials merits further investigation.

The physical contrast between the cheeses, as observed organoleptically, was confirmed to some extent by the scanning electron micrographs shown in Figure 3. The semi-hard, crumbly sodium caseinate cheese had the casein in dense aggregates, but the casein in the calcium and potassium caseinate cheeses had coalesced to give thin strands or plates. This difference suggests that a knife would slide more

Table 2. The effect of type of caseinate and maturation time on the coarseness of Feta-style cheeses.

Type of cheese/ time in days	Coarseness 1	14	28
Sodium caseinate	14.9	7.6	5.5
Calcium caseinate	6.1	5.0	4.9
Potassium caseinate	6.0	4.5	4.4
Gibna Baida	—	—	4.3

easily through the latter than the cheese made with sodium caseinate.

Similarly, the coarse strands of casein shown in Figure 3 (a) would allow the cheese to crumble into small pieces under pressure from the fingers, while the 'plates' of casein in Figure 3 (c) would merely compress; a view that was reflected in the contrasted behaviour of the two cheeses.

Clearly, therefore, the type of caseinate can have a marked effect on the structure of a finished cheese made by direct recombination. The system could be further refined and cheeses tailored for specific markets.

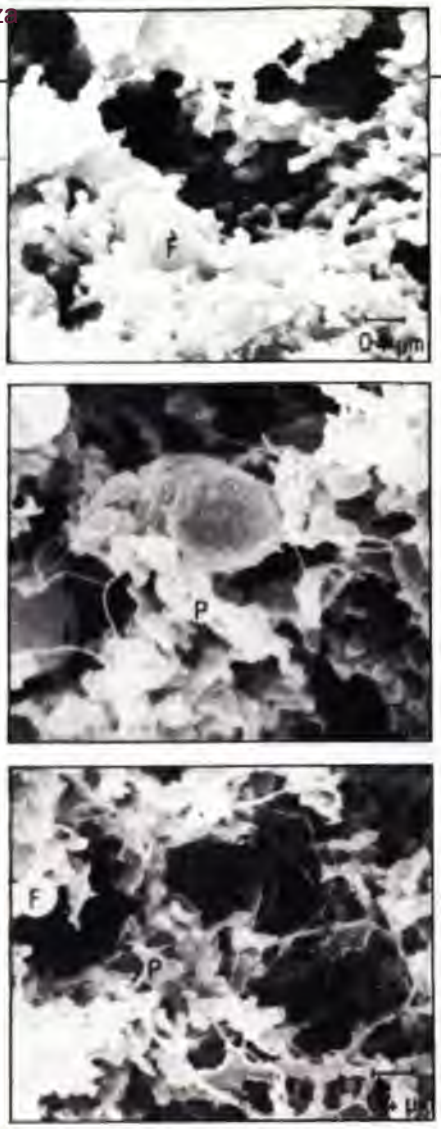


Figure 3. Scanning electron micrographs of one month-old cheeses made with (a) sodium (b) calcium and (c) potassium caseinate showing the fast globules (F), protein matrix (P) and aqueous phase (A).

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The influence of the casein:fat ratio in sheep's milk on the chemical composition and sensory properties of nabulsi cheese

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The organoleptic properties of nabulsi cheese, which is widely popular in Jordan, tend to vary from one market sample to the next, and differences in the chemical composition of the cheese milk were suspected. Deliberately altering the casein:fat ratios in Awassi sheep's milk influenced the texture of, and hence consumer reaction to, nabulsi cheeses made from the different milks. However, the use of a starter culture including Lactococcus lactis and L cremoris, rather than the natural microflora of the milk/dairy, produced cheeses that were perceived to be too harsh in flavour. It was concluded that the between-batch variability observed in the marketplace was likely to be introduced by both the uncontrolled nature of the bacterial populations in the milk and compositional differences.

Introduction

White brined cheeses are popular throughout the Mediterranean and Middle Eastern countries, and storage in brine (4–16%) has evolved as the most convenient system for distribution in such warm climates (Caric, 1987). In Jordan a boiled, white brined cheese, nabulsi, named after the town of Nablus on the West Bank, is the main cheese produced along traditional lines. It has a mild flavour and is widely consumed at breakfast or used in the manufacture of local desserts (Humeid and Tukan, 1986).

Traditionally, nabulsi was made from raw sheep's milk, but nowadays cheese on the open market must be made from pasteurized milk. Blends of cows', goats' and sheep's milk are used by some farmers to expand levels of production but, as the volume of nabulsi in the marketplace has increased, so have complaints about undesirable changes in quality, particularly with respect to texture.

Seasonal variations with respect to the fat and casein contents of sheep's milk have been cited as one possible cause, and it was suggested that if the optimum casein:fat ratio for the production of nabulsi cheese could be established, as it has been for cheddar cheese (eg, Chapman, 1981, McIlveen & Strugnell, 1990), then the organoleptic properties of the retail product might be less subject to fluctuation. Alternatively, the uncontrolled nature of the microflora could be the source of inter-batch variation, and hence the specific aims of this project were to: (i) study the effect of different casein:fat ratios on the chemical composition and sensory properties of nabulsi cheese made from Awassi sheep's milk; and (ii) determine the influence of a starter culture on the composition and properties of the same cheeses.

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Materials and methods

All the milk came from one herd of Awassi sheep in the El-Yadudah area near Amman; the sheep were fed on dry pasture throughout. Samples of milk were collected at monthly intervals between February and August 1993 for routine analysis of fat and casein to establish the extent of natural variations, and during July for the programme of cheesemaking.

Preparation of the starter culture

A freeze dried Dri-Vac culture (Type 0-180) from Chr Hansen's Laboratorium, Denmark, was used to manufacture some of the test cheeses; the culture consisted of *Lactococcus lactis* ssp *lactis* (2–5%) and *L lactis* ssp *cremoris* (98–95%). After the initial resuscitation stage, laboratory cultures were prepared by inoculating (1%, v/v) sterile reconstituted skim milk (100 ml of 10% total solids, w/v) and incubating at 30°C until the milk had just coagulated. Routine tests for purity and activity were carried out as recommended by Harrigan and McCance (1976), and larger volumes for addition to the cheese milk were produced according to the procedure of Tamime (1990).

Preparation of the cheese milk

Raw sheep's milk was warmed to 40°C and then filtered through cheesecloth before pasteurizing at 63°C for 30 min. After cooling to 38°C the cream was separated using an Alfa-Laval Type 24 S Separator, and the fat contents of the cream and skim milk streams

determined by the Gerber method. Once the casein content of the original pasteurized milk had been determined, the fat contents necessary to give ratios of casein: fat of 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 were calculated. The weights of whole milk and cream or skim milk to give the desired values of fat in the cheese milk were calculated using Pearson's Square Method (Scott, 1986).

Procedure for cheesemaking

Sufficient milk to make a block (1 kg) of cheese was poured into a stainless steel container equipped with a slow-speed agitator and standing in a temperature controlled water bath. $CaCl_2$ (0.02%, w/v) and 0.02% of standard rennet (diluted prior to addition to the milk) were added, and the milk held at 32°C for around 1 hour to coagulate; this slow rate of gel formation was a reflection of the reduced microflora consequent upon pasteurization.

When starter cultures (1%, v/v) were used the milk was ripened to pH 6.35–6.4 prior to the addition of the $CaCl_2$ and rennet.

Once the coagulum had formed, it was cut into 1 cm^3 cubes, and allowed to stand for 15–20 min to encourage expulsion of the whey. The curds and whey were then poured into a stainless steel mould lined with cheesecloth for an initial period of free drainage.

The cheese was pressed in two stages, ie, 0.4 MPa for 30 min followed by 0.8 MPa for 2 hours. After the second pressing the fresh cheese was cut into pieces of 5 × 4 × 2 cm, and coarse salt sprinkled over the surfaces. Following storage at 5°C for 24 hours all the pieces of cheese from one original block were weighed to obtain a measure of yield—kg of edible cheese/kg of fat in the original milk—and subsamples were taken for immediate chemical analysis

Thereafter, the pieces of cheese were carefully placed into glass containers and covered with brine (15%). After 2 days in store the pieces were removed from the containers and boiled in brine for 5–7 min, ie, until the surfaces of any given piece had acquired a thin skin of melted cheese. The pieces of boiled cheese were returned to the brine (15%) and stored for 1 month prior to the second group of analyses.

Procedures for testing the milk and cheese

(a) Chemical analysis

The milk was brought to the laboratory in churns and, after thorough stirring with a plunger, duplicate samples (200 ml) were removed with a dipper for the following analyses: (i) the fat content was determined by the Gerber method (Case, 1985); this method was employed for the cheeses also; (ii) the protein and casein contents of the sheep's milk were estimated by the formol titration method (Roeper, 1974).

For examining the cheeses, a subsample (approx 200 g) was removed from the brine and allowed to drain for 1–2 hours on clean filter paper placed inside a closed container. The entire sample was coarsely ground with a pestle and mortar to provide a homogeneous mass from which duplicate aliquots could be removed for the desired analysis, namely: (i) the protein contents of the cheeses were determined by the colorimetric method described by Case (1985), and the pH by the British Standard Method (BSI, 1976); (ii) the moisture, NaCl, lactose (colorimetric method) and calcium contents were assessed by the methods of Egan *et al* (1981); and (iii) the titratable acidity was determined by titration using phenolphthalein as the indicator (IDF, 1969).

(b) Microbiological analysis

In order to confirm the general microbial pattern within the cheeses a number of 'pieces' from a given batch of cheese were cut up with a sterile knife and a composite sample (approx 50 g) was collected into a sterile Stomacher Bag. A sterile solution of sodium citrate (450 ml, 2%) was poured into the bag, and the sample macerated for about 2 min. Serial dilutions down to 10^{-6} were prepared in sterile peptone water (9 ml, 0.1%), and duplicate aliquots were used to obtain the following information: (i) total colony count on Yeastrel milk agar—incubation at 32°C for 48 hours; (ii) yeast and mould count on potato dextrose agar with the pH adjusted to 3.5 with lactic acid—incubation at 25°C for 3–5 days; (iii) coliform count on violet red bile agar—incubation at 32°C for 24 hours (Marshall, 1992); and (iv) differential count for *L lactis* ssp *lactis* and *L lactis* ssp *cremoris* on spread plates of arginine tetrazolium agar—incubation at 30°C for 24–48 hours (Harrigan and McCance, 1976).

(c) Sensory evaluation

For tasting purposes the cheeses were grouped initially on the basis of the presence or absence of a starter culture and within each of these two groups were cheeses made from each of the milks with different casein: fat ratios. Each cheese within a group was given a random code, and 30 untrained panellists were asked to taste the cheeses in a preset sequence and rank them in order of decreasing preference (Amerine *et al*, 1965), ie, the 'best' cheese received 1 point and the 'worst' 6 points. Once the initial selections had been made, a group of 60 assessors participated in a paired preference test to determine the impact of the microflora on overall quality; this test involved only the 'best' cheeses from each group.

The data were analysed by analysis of variance (ANOVA) (SAS, 1985), and a

TABLE 1
Mean values (g/100 ml) for the major constituents of milk taken from a flock of Awassi sheep in Jordan; each value is the mean of eight readings, ie, duplicate samples taken four times each month

Month	Component			
	Protein	Fat	Casein	C/F ratio
February	6.7	5.2	5.7	1.1
March	6.6	5.6	5.6	1.0
April	6.7	6.5	5.7	0.9
May	6.7	7.9	5.7	0.7
June	7.4	6.7	6.3	0.9
July	7.2	6.4	6.1	1.0
August	6.6	8.6	5.6	0.7

Student *t* test confirmed the significant difference between cheeses made with and without starter cultures.

Results and discussion

The average values for protein, casein and fat in Awassi sheep's milk over the period from February to August 1993 are shown in Table 1; the range of protein contents was in line with those quoted by Epstein (1985). Although the C/F ratio fluctuated by only 0.2 about a mean of 0.9, in other situations more extreme elevations in fat contents (up to 11.8% in some flocks of Awassi sheep; Epstein, 1985) could reduce the C/F ratio to around 0.5.

Such seasonal variations in the C/F ratio might well be expected to alter the sensory properties of the endproduct, and the data presented in Tables 2 and 3, confirm that the C/F ratio in the original milk had a significant impact on both cheese composition and yield. In particular, the yields (kg of mature cheese/kg of fat in the cheese milk) increased significantly ($p \leq 0.05$) as the C/F ratios increased, while moisture content in the same cheeses decreased by over 10%. This pattern was in agreement with the results of Shaker *et al* (1987). In fact, some of the values for moisture content are even above the Jordanian Standards (1991), which stipulate a water content for nabulsi cheese of <50%. It is suggested that the higher moisture content arises because the elevated fat levels of the cheese milk interfere with the rate of syneresis. Typical market samples of nabulsi cheese gave a similar spread of values from 36.1–51.0% moisture (Humeid and Tukan, 1986, 1991), and it could be that variations in the C/F ratios of the milk supplies were, at least in part, responsible.

The fat and protein contents of the experimental cheeses increased, as would be expected (Ng-Kwai-Hang *et al*, 1987), as the C/F ratio approached 1.0 and the relative moisture contents of the cheeses decreased. These changes will have a direct bearing on the texture of the retail cheese. However, the significant effect ($p \leq 0.05$) of the starter culture on the relative levels of fat in the finished cheeses was not anticipated. Thus the calculated values (means) for fat in dry matter fell from 53.2% in non-starter cheeses to 42.7% for the cheeses with added starter culture, and the reason(s) for the poor recovery into the latter cheeses was not established.

The salt contents followed the pattern of the water, although the range of values was restricted compared with the salt contents of typical market nabulsi cheeses, which ranged from 5.7–13.3% (Humeid and Tukan, 1986). The diffusion of sodium ions into the cheese could also explain, at least in part, the decrease in calcium concentrations during storage (see Table 3). Thus while the decrease in pH causes some of the calcium phosphate associated with the casein micelles to leach into the whey (Abd El-Salam *et al*, 1976; Shammet *et al*, 1992), the process of ion exchange may be equally important in brined cheeses.

TABLE 2
Effect of different casein:fat ratios on the chemical composition and yield of nabulsi cheeses manufactured with or without a standard starter culture (see text for further details)

C/F ratio	Storage	Yield (kg/kg of fat)		Protein (%)		Moisture (%)		Fat (%)	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
0.5	Nil	2.4	2.4	17.0	19.0	52.7	55.4	26.5	19.4
	1 month	1.8	1.9	15.8	17.4	51.2	53.3	27.6	20.1
0.6	Nil	2.7	2.8	17.4	22.0	49.0	53.2	28.1	19.9
	1 month	2.2	2.2	15.8	20.1	47.3	50.3	28.9	20.5
0.7	Nil	3.1	3.1	19.3	22.4	48.1	51.2	28.4	20.4
	1 month	2.5	2.5	17.7	20.7	44.5	48.1	29.9	21.9
0.8	Nil	3.5	3.5	19.3	22.7	47.5	50.0	27.5	19.6
	1 month	2.8	2.8	17.3	21.0	43.5	49.3	29.3	19.8
0.9	Nil	3.7	3.8	20.6	23.1	44.3	46.6	27.7	20.7
	1 month	3.0	3.1	19.7	21.4	42.5	44.1	28.9	21.5
1.0	Nil	3.9	4.1	21.4	23.4	43.6	44.6	28.1	21.3
	1 month	3.2	3.3	19.9	21.7	39.3	40.5	29.7	21.9

(a) = Cheeses manufactured without added starter culture.
(b) = Cheeses manufactured with a blend of *L lactis* and *L cremoris*.

TABLE 3
Effect of different casein:fat ratios on selected chemical properties of nabulsi cheeses manufactured with or without a standard starter culture (see text for further details)

C/F ratio	Storage	Titratable acidity (%) (a)	(b)	Salt (%) (a)	(b)	Calcium (mg/100 g) (a)	(b)	Lactose (g/100 g) (a)	(b)	pH (a)	(b)
0.5	Nil	0.19	0.36	3.9	4.4	158.2	98.5	5.2	2.9	6.7	5.6
	1 month	0.29	0.41	8.8	9.6	97.0	28.6	0.5	0.4	5.6	5.1
0.6	Nil	0.19	0.35	3.2	3.5	163.3	94.8	4.0	3.5	6.7	5.6
	1 month	0.31	0.41	8.5	9.2	99.5	24.0	0.5	0.4	5.6	5.2
0.7	Nil	0.18	0.36	3.1	4.2	165.2	97.1	3.8	3.0	6.7	5.6
	1 month	0.30	0.43	7.6	8.7	96.0	24.0	0.5	0.6	5.6	5.2
0.8	Nil	0.21	0.32	3.4	3.9	163.4	93.4	4.1	3.3	6.7	5.6
	1 month	0.32	0.42	7.5	8.7	89.6	23.0	0.5	0.7	5.6	5.2
0.9	Nil	0.20	0.34	3.2	3.5	163.6	87.8	3.5	2.5	6.7	5.6
	1 month	0.32	0.46	6.4	7.4	91.2	21.5	0.4	0.7	5.6	5.2
1.0	Nil	0.22	0.33	3.1	3.4	155.5	86.2	3.4	2.6	6.7	5.6
	1 month	0.31	0.45	6.3	6.9	87.2	20.2	0.5	0.7	5.6	5.2

(a) = Cheeses manufactured without added starter culture.
(b) = Cheeses manufactured with a blend of *L. lactis* and *L. cremoris*.

TABLE 4
Typical total colony counts for the two groups of cheeses, together with an indication of the survival of the starter culture—the original cheese milk had, on average, counts for both species of *Lactococcus* in excess of 10×10^6; counts for coliforms and yeasts and mould never exceeded <10/g

Time	Total colony count (cfu $\times 10^6$/g) (a)	(b)	*L. lactis* (cfu/g) (a)	(b)	*L. cremoris* (cfu $\times 10^6$) (a)	(b)
Before storage	25	24		4000		11
After storage	159	160		<30		

(a) = Traditional cheese.
(b) = Cheese made with a starter culture.

The impact of starter culture activity (see Tables 2 and 3) was significant ($p \leq 0.05$). The increased levels of acidity were expected, and it may be that the values were elevated by the residual proteolytic and lipolytic activity of enzymes from the starter culture (Saleem, 1979; Abou-Donia, 1981). Thus although the combined impact of boiling and brining appears to lead to rapid death and autolysis of the starter bacteria (see Table 4), the sensory analysis revealed that the influence of the culture was readily discernible. The low levels of residual lactose in the two groups of cheeses probably reflect the activities of the adventitious bacteria (see Table 4) but, as pasteurization severely reduced the total counts compared with most white brined cheeses (Haddadin, 1986), these casual contaminants had no adverse effect on the flavour of the experimental cheeses.

The results of the ranking experiment indicated that the preferred cheeses had C/F ratios of 0.7, while the cheeses at the two extremes were rated as 'poor' (see Table 5). It was suggested that texture was the main reason for this discrimination in that the high moisture content at C/F 0.5 gave too soft a cheese, while the higher casein content (C/F 1.0) gave rise to a somewhat tough product.

The significant ($p \leq 0.05$) preference for the non-starter cheese was, as revealed by the paired preference test, clear cut, and the sharper, stronger flavour produced by the lactococci was not appreciated. As nabulsi cheese is often used in desserts, the preference for a mild flavour may not be surprising. Nevertheless, the result does suggest that the uncontrolled microflora of many market cheeses could have contributed to the perceived lack of consistency between batches.

TABLE 5
Results of the sensory evaluation of mature nabulsi cheeses made from pasteurized milks with different casein:fat ratios and with and without a starter culture. The rank totals were obtained by adding the ranking scores from 'good' (1) to 'poor' (6) given by 30 panellists to cheeses made with the C/F ratios indicated; low numbers indicate a high degree of acceptability. The two best cheeses (C/F 0.7, with/without starter culture) were offered to 60 panellists for appraisal for overall preference

C/F	Cheeses without starter: rank totals	Cheeses with starter: rank totals
0.5	149	172
0.6	96	81
0.7	35	44
0.8	76	87
0.9	121	137
1.0	141	108
Overall preference (scores out of 60)		
	39	21

Conclusion: the most highly rated cheese was made from milk with a C/F ratio of 0.7 and no added starter culture

Overall, it is clear from this study that both the casein:fat ratio of the raw sheep's milk and the nature of its microflora can have a dramatic effect on the chemical composition and sensory properties of the final cheese. Whether or not the economics of production will support improved control over the conditions of production and maturation remains to be seen. Nevertheless, consumer demand for consistant, mild flavoured cheeses is unlikely to be met by traditional techniques alone, and it may be that cooperative ventures will offer a way forward.

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The isolation of salt-tolerant lactic acid bacteria from ovine and bovine milks for use in the production of nabulsi cheese

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Nabulsi cheese is one of the most popular white brined cheeses produced in Jordan and, traditionally, no starter cultures are employed for ripening. However, the resultant high pH means that spoilage problems arise in cheeses stored at ambient temperatures. Direct acidification or the use of commercial starter cultures have an adverse effect on flavour, but cultures derived from lactic acid bacteria isolated from local milks gave rise to cheeses that were as acceptable as the traditional products. In addition, the pHs of the cheese brines were in the range associated with stability during storage (pH 5.1–5.3), and hence it is suggested that these locally derived cultures could be employed by the industry to manufacture a nabulsi cheese from pasteurized milk that should: (a) be free from pathogens; (b) not deteriorate in-store at ambient temperature; and (c) have a flavour profile similar to traditional nabulsi cheese.

INTRODUCTION

White brined cheese is an important traditional food in the Mediterranean region,[1] and the range includes feta in Greece, domiati in Egypt, halloumi in Cyprus and Lebanon and teleme in Romania.[2] The equivalent cheese in Jordan is nabulsi,[3] and it is usually produced in springtime to coincide with the availability of ovine and caprine milk. The cheese is mainly consumed at breakfast or as a snack, or it is used as an ingredient in the preparation of some local traditional dishes and sweets, especially kunafa.[4]

Traditionally, the production of nabulsi cheese depends on a method in which no starter culture is used, and this method can be summarized as follows: ovine milk, or a mixture of ovine and caprine milks, is heated to about 35°C and coagulated with rennet. The curd is pressed in cheesecloth into rectangles with a height of about 2 cm. The rectangles are then cut into pieces (about 4 × 8 cm) and sprinkled with salt. The fresh cheese can be consumed as such, but it has very limited keeping qualities even under refrigeration. Therefore, it is common practice to boil pieces of cheese in brine containing 20% (w/v) NaCl, along with the spices mastic (*Pistacia lentiscus*) and mahaleb (*Prunus mahaleb*), which are hung in the boiling brine in a small cheesecloth bag. The boiling process serves not only to improve the shelf-life of the product, but also to provide texture (firmness) to the cheese, and it is complete when the pieces of cheese become soft and float to the surface of the brine; this process usually requires 5–15 min. The pieces are then taken out of the brine, placed on a flat surface and reshaped by slight pressing. After cooling, the cheese is placed in firmly closed cans (10 or 20 l capacity) containing some of the brine in which the cheese was boiled. Thus nabulsi cheese depends on the boiling process and the high salt concentration of the brine to keep it stable during non-refrigerated storage for up to one year.

However, although nabulsi cheese is stored in a brine with a high salt concentration, usually >20% (w/v) NaCl, spoilage can still occur and cause heavy losses to the producers. Some 'on-farm' trials involving the direct acidification of the storage brine with lactic acid showed promising results with respect to the shelf-life of the product, and hence it was suggested that the same effect might be achieved by producing a more acid curd during the actual cheesemaking process.

The potential of this latter option was examined by Haddadin *et al*[4] employing a mixed culture of *Lactococcus lactis* subsp *lactis* and *Lactococcus lactis* subsp *cremoris* to generate the lactic acid and, although this was successful in lowering the pH of the cheese, consumers found the flavour of the cheese a little harsh. However, if a new starter culture could be developed based upon species normally present in the raw milk used for producing nabulsi, then it might be possible to generate the necessary acidity without changing the typical flavour anticipated by local consumers.

Consequently, the aims of this present study were to (a) examine samples of local bovine

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and ovine milks for lactic acid bacteria (LAB) that might be employed as starter cultures; salt-tolerance was employed as one criterion for selection, in the expectation that the bacteria might continue to secrete lactic acid during the overnight hold in brine that has now, in many factories, replaced the traditional dry salting procedure; and (b) determine whether the use of these 'local' starter bacteria would provide the acidity that appears necessary to reduce spoilage but at the same time give a product that would be as acceptable to consumers as the traditional cheese.

MATERIALS AND METHODS

Isolation and identification of salt-tolerant LAB from fresh milk

Fifteen samples of bovine milk and 10 samples of ovine milk were collected in sterile bottles from individual farms under aseptic conditions and transported to the laboratory in a 'cool-box' containing ice. In the laboratory, 1 ml from each sample was transferred immediately into tubes containing 9 ml of sterile (121°C, 3 min) skim milk (RSM) reconstituted from powder (Reglait, France) at 11% (w/v) and containing 5% NaCl (w/v). After two and seven days of incubation at 35°C, serial dilutions (down to 10^{-7}) were made from each inoculated tube using peptone water (0.1%, w/v) as the diluent. Duplicate pour plates of bromocresol green whey agar (BGWA)[5] were then prepared from dilutions 10^{-6} and 10^{-7}, and incubated at 35°C for 72 h. Representative colonies from BGWA were inoculated into RSM (11%, w/w) and incubated at 35°C either until a curd was formed or for 72 h. A loopful from each culture was then streaked onto BGWA, and single colonies from each BGWA plate were subcultured twice to obtain a pure isolate; these cultures were identified according to the standard texts,[6-9] but with the nomenclature revised in line with Hammes and Vogel,[10] Teuber[11] and Devriese and Pot[12]; a list of the tests performed to identify the individual species is given in Table 1.

Effect of salt-tolerant cultures on the pH of RSM

Loopfuls from the stock cultures of the isolated species were inoculated into 10 ml of sterile RSM (11%, w/v) containing 5% sodium chloride, and the tubes were incubated at 35°C for 72 hours. The pH of each culture was measured using a pH meter (Hanna HI-8519, Milan, Italy) after 24 h and then at the end of the incubation (72 h).

Production of nabulsi cheese using the salt-tolerant cultures

Ovine milk was obtained from a local dairy plant, and the pH was determined using a pH meter (Hanna HI-8519). A white brined cheese of the nabulsi type was then produced along the lines described earlier[4,13]; the stages of production are summarized in Fig. 1. Seven batches of cheese (treatments) were made in all, including one 'control' cheese made without starter culture, and the six cultures used are listed in Table 4; the entire trial was repeated on three separate occasions.

Examination of the cheese

pH of nabulsi cheese

Cheese samples were taken from each treatment directly before brining, after boiling and after one week of storage at 30°C. The pH values of the samples were determined using a pH meter (Hanna HI-8519) according to the method of the BSI,[14] in which a well homogenized cheese sample (10 g) is mixed with 6 ml deionized water to form a fine suspension into which the electrode of the pH meter can be immersed.

pH of the storage brine

Brine samples were taken for each treatment after the boiling stage and after it had been used to store the cheese for one week at 30°C.

TABLE 1

Physiological characteristics of the salt-tolerant species of *Lactobacillus*, *Lactococcus* and *Enterococcus* species isolated from samples of local bovine and ovine milk[6-9]

Species	Carbohydrates fermented																			
	Ar	*Ce*	*Aesc*	*Fru*	*Gal*	*Gl*	*Lac*	*Malt*	*Manni*	*Manno*	*Mele*	*Meli*	*Raf*	*Rha*	*Ribo*	*Sal*	*Sor*	*Su*	*Tre*	*Xyl*
Lb paracasei	–	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–
Lb rhamnosus	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–
L lactis subsp *lactis*					+		+	+							+					
E faecalis	–								+								+	+		
E faecium	+								+								–	+		
E durans	–																	–		
E avium	+								+								+	+		
E pseudoavium	–								+								+	–		
E hirae	–								–			+	+				–	+		

Ar = arabinose; Ce = cellobiose; Aesc = aesculin; Fru = fructose; Gal = galactose; Gl = gluconate; Lac = lactose; Malt = maltose; Manni = mannitol; Manno = mannose; Mele = melezitose; Meli = melibiose; Raf = raffinose; Rha = rhamnose; Ribo = ribose; Sal = salicin; Sor = sorbitol; Su = sucrose; Tre = trehalose; Xyl = xylose.
+ = positive; – = negative; no symbol = not tested.

Pasteurize sheep's milk (63–65°C/30 min)
Cool to 35°C
Add $CaCl_2$ (0.02%, w/v) and starter culture (2%)
Keep at 35°C for 30 min
Addition of rennet and coagulation over 30 min at 35°C
Cut into cubes, *circa* 2 cm
Rest for 15 min
Drain through cheesecloth placed in a rectangular stainless steel frame (5 × 37 × 39 cm)
Press the curd against the bottom plate at low and then high pressure
Cut and shape
Cut the flat 'plate' of cheese into pieces (5 × 3 × 1.5 cm)
Hold overnight at room temp in brine in 15% NaCl (w/v)
Boil in the same brine for 5 min
Store at 30°C for 2 weeks in 500 ml jars

Fig. 1. The various stages in the production of nabulsi cheese from ovine milk using cultures of salt-tolerant LAB isolated from local supplies of bovine or ovine milk.

The pH value of each sample was determined using a pH meter.

Determination of sodium chloride

The sodium chloride contents of both the cheese and the brine samples were determined according to the method described by AOAC.[15]

Sensory evaluation

A tentative evaluation of consumer reactions to the test cheeses was carried out using a hedonic scale to investigate the degree of preference.[16] Each panelist expressed his/her degree of 'like' or 'dislike' by completing a standard form, and nine untrained panelists from the staff of the Department of Nutrition and Food Technology of the University of Jordan took part in the evaluation. The panelists were requested to taste each of the seven cheeses separately and to score each cheese without reference to the other samples. As the number of panelists was too low to provide a true picture, the results of the sensory examination were not analysed for statistical significance and were used to indicate a 'likely trend' only.

TABLE 2

Changes in the pHs of RSM containing 5% NaCl after inoculation with salt-tolerant species of LAB isolated from raw bovine (B) or ovine (O) milk from different farms in Jordan; incubation at 35°C for the times indicated

Culture	*24 hours*	*72 hours*
Lb rhamnosus (B)	5.4	4.4
Lb paracasei (O)	5.1	4.1
L lactis subsp *lactis* (B)	5.7	4.6
E hiare (O)	5.5	5.3
E faecalis (B)	5.4	5.0
E faecium (O)	5.5	5.1
E durans (O)	5.6	5.1
E pseudoavium (O)	5.6	5.2
E avium (O)	5.5	5.3

TABLE 3

Changes in the pH of the nabulsi cheese produced from ovine milk (original pH 6.6) and the keeping brine as induced by the bacteria indicated; all the species were isolated from ovine or bovine milk of local origin

	Cheese			*Brine (15% NaCl)*	
Culture	*At cutting*	*After boiling*	*At 7 days*	*After boiling*	*At 7 days*
Lb paracasei	6.2	5.8	5.8	6.6	5.5
Lb rhamnosus	6.2	5.8	5.7	6.5	5.1
L lactis subsp *lactis*	5.8	5.3	5.3	5.7	5.1
E faecalis	5.8	5.6	5.5	6.3	5.2
E faecium	6.1	6.0	5.8	6.3	5.6
E durans	6.1	6.1	5.9	6.1	5.3
Control	6.4	6.4	6.1	6.7	5.7

Results and discussion

Twelve out of 15 samples of bovine milk contained LAB which were able to grow in RSM containing 5% NaCl, and these isolates were identified according to the scheme shown in Table 1. Most of the isolates belonged to the genus *Enterococcus* (three species were identified), while one species of salt-tolerant *Lactococcus* spp and one of *Lactobacillus* were represented less frequently. As with the samples of bovine milk, most of the isolates from ovine milk belonged to the genus *Enterococcus*, but there were some interesting differences in species composition. Thus while *Enterococcus faecium* was prominent in sheep milk, salt-tolerant strains of *Enterococcus faecalis* were found in bovine milk. Similarly, *Lactococcus lactis* subsp *lactis* was isolated only from bovine samples, while the presence of *Lactobacillus paracasei* (formerly *Lactobacillus casei* subsp *pseudoplantarum*) and *Lactobacillus rhamnosus* (formerly *Lactobacillus casei* subsp *rhamnosus*) altered with the origin of the milk. Such salt-tolerant LAB were also isolated from raw salted milk used in the production of domiati cheese, a brined white cheese made from milk which is salted directly before renneting.[17]

Table 2 shows changes in the pH of the RSM (11%, w/v) that resulted from the growth of the salt-tolerant LAB isolated from the fresh milk samples. The growth of *Lb paracasei* was the most active as indicated by the drop in the pH, followed by *Lb rhamnosus* and *L lactis* subsp *lactis*. Therefore, these three organisms were selected for cheesemaking, along with three species of *Enterococcus* (see Table 4) that have a history of occasional usage in the dairy industry.

The addition of different cultures to the ovine milk (pH 6.6) resulted in a lowering of the pH of the cheeses during the different stages of production (Table 3) compared with cheese made without any starter culture (control). The pHs of the brines were similarly lowered by the different cultures, with *Lb rhamnosus* and *L lactis* subsp *lactis* being particularly active. Whether or not the contrasted levels of acidity in the brine/cheese is a reflection of the relative salt-tolerance of the strains is not clear, but it is notable that the pHs of the cheeses after boiling are, in most cases, lower than the values recorded before overnight brining. However, a similar degree of acidification was reported using a culture

with low salt tolerance,[4] so that acid production during the brining stage may depend upon residual enzyme activity rather than active bacterial metabolism; the thickness of the cheese pieces (1.5 cm) suggests that salt from the brine (15% w/v NaCl) must readily permeate the cheese and inhibit most microbial action. Nevertheless, the high salt contents in the cheese (around 7.0% w/w) and the brine, together with the increase in acidity vis-à-vis the control, would be expected to inhibit most spoilage organisms, and it could be that the use of a selected starter culture would give cheesemakers the protection that they are seeking.

If this suggestion is correct, then it is relevant that, after two weeks, no significant differences were observed between the sensory scores for cheeses produced with the various species of salt-tolerant bacteria (see Table 4). It was notable though that *L lactis* subsp *lactis* gave rise to a cheese that was 'least liked', which tends to confirm an earlier view[4] that standard mesophilic cheese starters may not be suitable, at least from an organoleptic standpoint, for manufacturing nabulsi cheese.

Thus it may be important that a number of salt-tolerant LAB were present in the indigenous microfloras of local ovine and bovine milks, and hence these bacteria might be expected to play an important role in the formation of the flavour and other sensory characteristics of the traditional dairy products made from fresh milk. In nabulsi cheese, for example, made from unpasteurized milk, these bacteria might act at all stages of processing that precede boiling, and hence it is not surprising that local consumers have acquired an appreciation of the flavours so generated. Consequently, the use of salt-tolerant starter cultures of the type isolated in this study could help to provide products with a high degree of uniformity and acceptability when using pasteurized milk. Further studies are needed to optimize the conditions for the action of these bacteria, especially with regard to length of time that the cheese is held in brine prior to boiling. This stage could be of paramount importance if the cultures are to generate the flavour and aroma typical of nabulsi, as well as the lactic acid necessary for stability during storage.

Whether or not enterococci should be employed as starter cultures is open to debate, for the alleged resistance of some strains of *E faecalis* and *E faecium* to penicillin[7] is a matter of some public health concern. The species of *Lactobacillus* are, however, likely to be widely acceptable and, as their performance during the present trial was better than the enterococci anyway, it is suggested that either isolate could be employed as a starter culture for the production of nabulsi cheese on a routine basis.

TABLE 4

Mean scores for the assessments of overall quality of the nabulsi cheeses made from pasteurized ovine milk and starter cultures of the species indicated; letters in parentheses indicate origin of culture (B = bovine, O = ovine). All cheeses were stored in brine (15% NaCl) for 2 weeks at 30°C prior to tasting. On the hedonic scale 1 = 'dislike very much' and 9 = 'like very much'

Culture	Overall score
Lb rhamnosus (B)	6.8
Lb paracasei (O)	6.7
L lactis subsp *lactis* (B)	6.3
E faecalis (B)	6.7
E faecium (O)	7.3
E durans (O)	7.5
Control (no starter)	7.5

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THE EFFECT OF SUB-CULTURING ON THE SPECIES COMPOSITION OF MULTIPLE-STRAIN CHEESE STARTERS

by

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ABSTRACT

Two multiple-strain, mesophilic starter cultures of commercial origin were subjected to routine sub-culturing in order to assess their potential stability. Acid production and species balance were monitored at both 22°C and 30°C, together with a vitality test under simulated cheesemaking conditions.

The results indicated that 22°C was the better temperature for maintaining cultures in skim-milk, but success of the technique depended on the strains present in the original culture; 30°C was better for cultures that were to be immediately employed for cheesemaking.

Introduction

Mesophilic lactic acid bacteria are widely employed in the manufacture of cheese, and the available commercial cultures are classified into single-, mixed-, and multiple- strain starters (Lawrence *et al.*, 1976).

It is the presence of the different strains that makes these latter cultures of especial interest to the industry (Robinson, 1981), and yet their behaviour within the confines of a creamery is still subject to some debate. If they are directly available for bulk starter production or direct-to-vat operation, then the quality of the end-product may be more important than the interactions of the micro-organisms *per se*. However, in many parts of the world, cultures can only be purchased occasionally, and the question arises as to whether multiple-strain starters can be subcultured successfully, and if so, what are are the optimum conditions for serial transfer. It was to answer these questions, at least in part, that this work was undertaken.

Factors influencing starter activity

The behaviour of an individual species often changes when it is grown in combination with others, and antagonistic and synergistic associations between lactic streptococci may lead, in mixed cultures, to the dominance of a particular species (or strain of that species) after two or three routine transfers (Reddy *et al.*, 1972; Collins, 1961; Vedamuthu *et al.*, 1966). Some fluctuations of this type result from the production of antibiotics (Babel, 1977; Collins, 1961), but, in the main, dominance is determined by differences in growth rate and/or relative viability subsequent to the stationary phase (Lawrence *et al.*, 1976).

These characteristics, together with the associated aspect of acid production, are influenced, according to Stadhouders (1974) and Davis (1965), by many factors including:

i) genetic characteristics of the individual strains;
ii) chemical composition of the milk, including the presence of stimulatory and inhibitory systems;
iii) presence of bacteriophage; and
iv) the system of culture transfer/growth adopted.

The potential role of the metabolic characteristics of starter organisms was demonstrated by Demko *et al.* (1972), who related the slow acid production of some variants of *Str. lactis* to an intracellular deficiency of fructose 1,6-diphosphate; the latter activates the lactic dehydrogenase system. The production of acid in milk depends also on the relative capacity of lactic streptococci to hydrolyse milk proteins, since normal milk contains limited quantities of assimilable nitrogen (Garvie, 1959; Stadhouders, 1961 and 1974). Evidence in support of this latter idea was obtained when a mixed population of thermophilic lactic acid bacteria was grown in milk to 10^6 -10^7 cells/ml., and then killed by heating. When a mesophilic starter was inoculated into this same milk, the rate of acid production increased *vis a vis* the control, and it was concluded that low molecular weight nitrogen compounds were the stimulatory factor (Sharpe, 1979), a view supported by the earlier work of Dahia and Speck (1964), as well as the reports by Prentice (1982) and Sponcet (1982).

Some important metabolic properties of the lactic acid bacteria of dairy starters are thought to involve plasmid

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genes. Specific components of the lactose metabolism system (multi-step process) are plasmid encoded (Anderson and McKay, 1977; LeBlanc *et al.*, 1979; Cords and McKay, 1974).

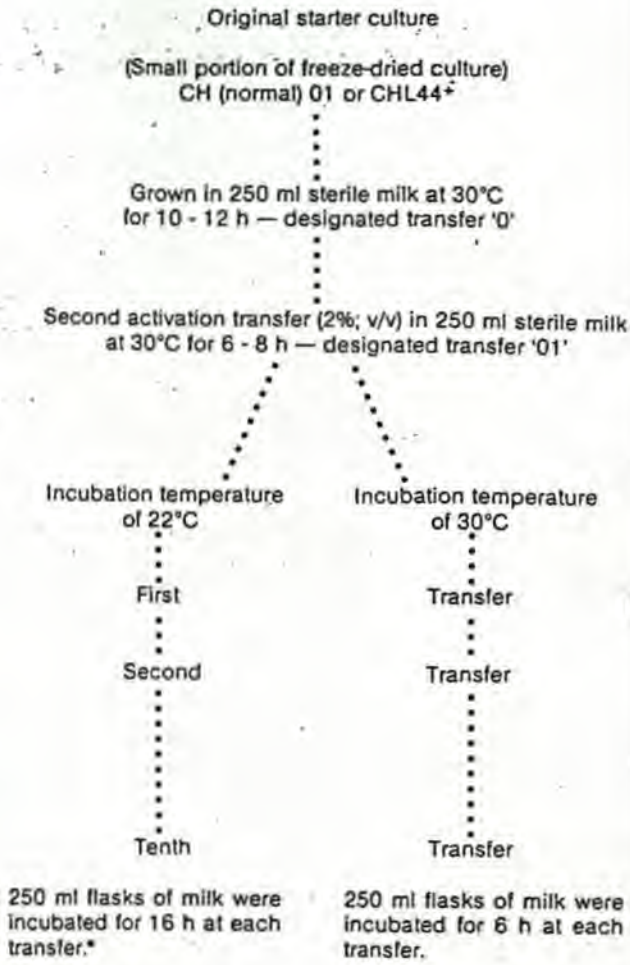
Proteinase negative variants of *Str. lactis* (C_2) were found to lack a 10 Md (mega dalton) plasmid as reported by McKay and Baldwin (1975).

The loss of proteinase acitivity in strains of *Str. lactis* was easily restored in contrast to strains of *Str. cremoris* (Larsen and McKay, 1978), and Kempler and McKay (1979) correlated the inability of variants of *Str. lactis* subsp. *diacetylactis* (DRC1, 18 and 16) to produce acetoin from diacetyl to the loss of a 5.5 Md plasmid.

It is also relevant that some strains of *Streptococcus* spp. tend to show reduced viability in the presence of high concentrations of lactic acid, and hence the age of a starter culture may deleteriously affect the quality of a cheese (Pearce *et al.*, 1973; McDowall *et al.*, 1960). The H_2O_2-CNS peroxidase system is similarly thought to be inhibitory to lactic mesophiles (Sharpe, 1979), although Stadhouders (1974) observed that some starters were, in fact, stimulated by peroxidase systems, and some strains produce detectable levels of H_2O_2 (Gilliland and Speck, 1969).

It is clear, therefore, that a number of factors can influence the growth of mesophilic lactic acid bacteria in milk, and that these factors will become acutely relevant during the sequential transfer of working cultures. This point is borne out, to some extent, by the controversy that exists concerning the optimum temperature for starter incubation. Thus, while Hammer and Babel (1957) suggested an incubation temperature of 21-22°C, Cogan and Condon (1971) indicated that a temperature not exceeding 27°C would be acceptable. However, Pack *et al.* (1968) stated that when 'aroma' bacteria, e.g. *Str. lactis* sub-sp. *diacetylactis* and *Leuconostoc cremoris* were present in mixed starters, their population was more stable at 30°C than at 21°C, and it is notable that many multiple-strain starters include these latter species.

It was decided, therefore, to investigate the effect of daily transfer at different incubation temperatures, namely 22 and 30°C, on the growth of the component micro-organisms of two multiple-strain starters, and to assess the possible influence of any observed changes on the rate of acid production by the resultant cultures; a vitality test under simulated cheesemaking conditions was performed, as well, after each transfer at the different incubation temperatures.



*To allow for continued activity in the refrigerator, 30 and 40 minute reductions in the final incubation times were given at 22 and 30°C respectively.

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FIGURE 1. Flow-diagram of the transfer programme employed

Materials and Methods

The two cultures employed during this study were standard, multiple-strain cheese starters, (CHL-N01 and CHL44-Chr. Hansens Laboratorium A/S, Denmark) and they were selected on the basis of:

i) consistent production of lactic acid on resuscitation from the freeze-dried state; and

ii) the high levels of *Str. cremoris* and *Str. lactis* relative to the aroma producing species.

The procedure for activation of the cultures is outlined in Figure 1 as is the overall plan of the experiment. Skim-milk powder (antibiotic free)

was reconstituted at a level of 9% (w/v) to provide a standard substrate, and the flasks of milk were sterilised in an autoclave at 115°C for 10 minutes. After cooling, the milk was held at 6°C prior to use; no flasks were retained for more than 4 days. The chilled milks were then inoculated as required by the experimental procedure, and tempered in a waterbath (45°C) for 3-5 minutes prior to transfer to an incubator (3°C) or waterbath (22°C). Duplicate flasks were employed throughout, and incubation times of 16 h (22°C) and 6 h (30°C) were retained as standard.

At the end of incubation, the test flasks were rotated gently to render the contents homogeneous, and one ml portions of the test milks were pipetted into 9 ml aliquots of Ringers solution (quarter-strength). These dilutions were mixed for 30 seconds with a 'Whirly Mixer' (Martley, 1972), and employed as the basis for an appropriate dilution series. Duplicate portions (1 ml) were extracted from the selected dilutions (10^{-5}, 10^{-6} and 10^{-7}), and pour plates were set up using Oxoid Milk Agar to obtain a total colony count, and Arginine Tetrazolium Agar (ATA) (Turner *et al.*, 1963) to provide a means of differentiation between *Str. cremoris* and the other species in the starters; all the plates were incubated at 30°C.

A collective enumeration of *Str. lactis* sub-sp. *diacetylactis* and *Leuconostoc cremoris* was carried out on Calcium Citrate Agar, using the modified method of Nickeles and Leesement (1964), and hence the recorded colony counts were obtained as follows:

Str. cremoris	i)	total number of arginine negative colonies on ATA;
	ii)	total colony count minus total number of arginine positive colonies on ATA (confirmation only);
Str. lactis	—	total number of arginine positive colonies on ATA minus total colony count (48 h) of 'aroma' bacteria on Citrate Agar;
Str. lactis sub-sp. *diacetylactis* and *Leuconostoc cremoris*	-	total colony count on Calcium Citrate Agar.

No attempt was made to differentiate between strains of any given species.

All counts were recorded in duplicate, and the results shown in Figure 3 are the average counts computed on the basis of two identical programmes (Figure 1).

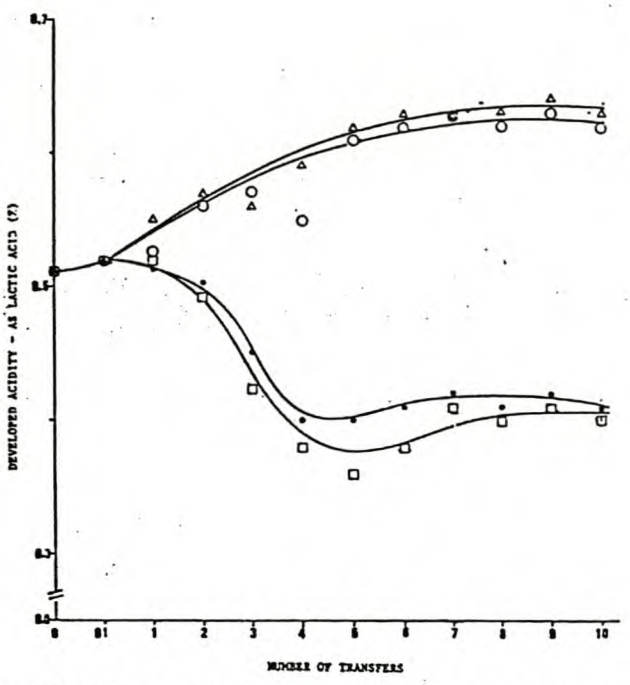

FIGURE 2. Developed acidity of cheese starter cultures with successive transfers at different incubation temperatures in sterile skim-milk. Initial total acidity of the sterile skim milk was 0.206%. Starter culture CHL-N01 at 22°C incubation temperature, Δ ; starter culture CHL-N01 at 30°C incubation temperature, □; starter culture CHL 44 at 22°C incubation temperature, o ; starter culture CHL 44 at 30°C incubation temperature, ●.

The development of acidity was monitored by determining the titratable acidity of duplicate samples of milk from the test flasks employing the method described by Robinson and Tamime (1976), and the results were recorded directly as 'percentage lactic acid'.

The vitality test was carried out using a modification of the procedure of Cox and Lewis (1972). In this present work, flasks (250 ml) of reconstituted whole milk (16% TS) were inoculated with starter culture at a rate of 1% v/v, and this procedure was repeated, for both cultures, after each transfer at the two temperatures of incubation. Rennet was added, and the titratable acidity (as % lactic acid) was recorded after six hours of incubation at 30°C.

Results and Discussion

The production of acidity is, of course, an essential feature of any starter culture for cheese, and it is clear that the ability of both starters to produce lactic acid is best maintained at 22°C (Figure 2). The fact that this facility is required at 30°C for the cheesemaking

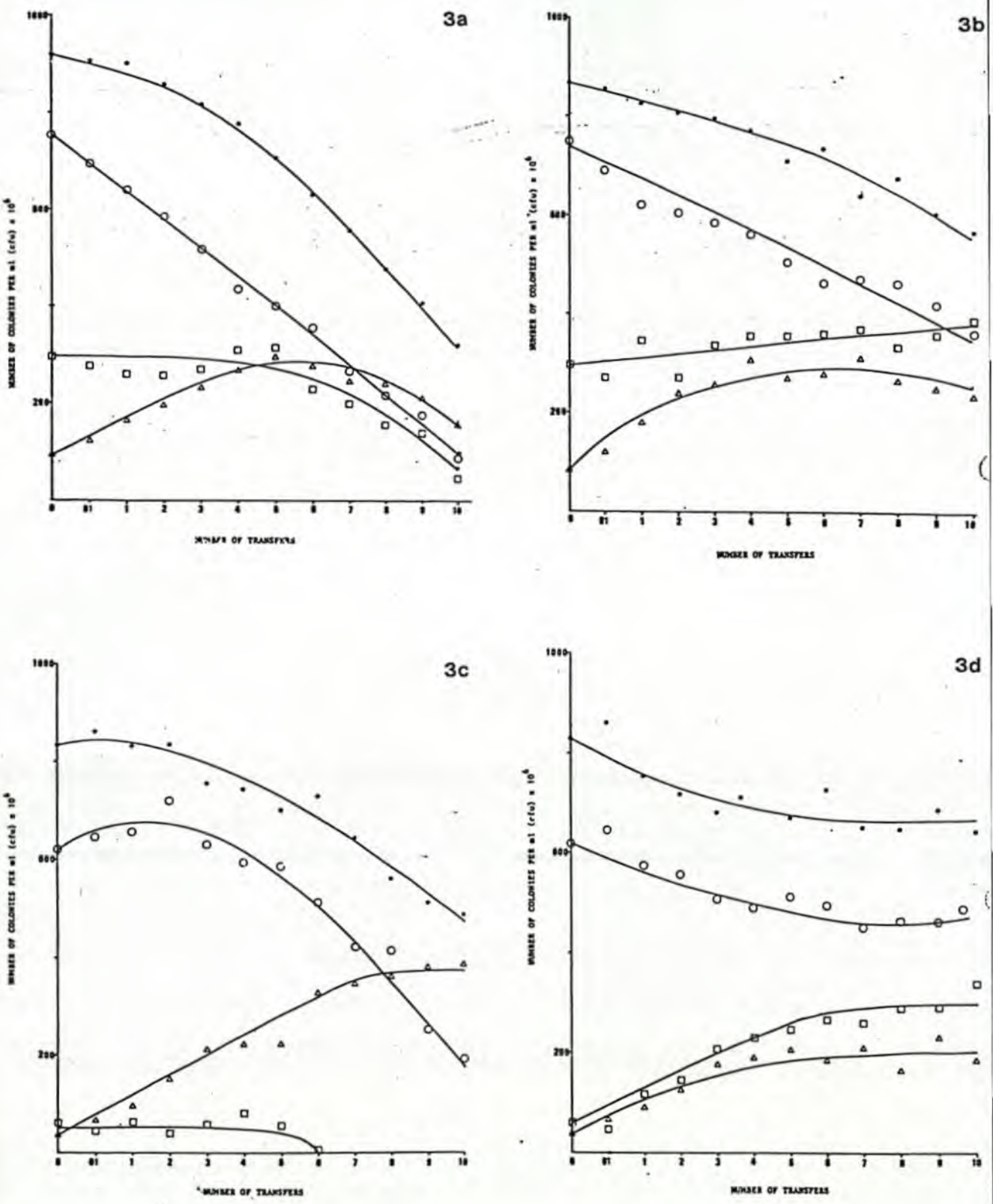

FIGURE 3. Populations of the component organisms of commercial cheese starter cultures during successive transfers in sterile skim-milk at two incubation temperatures. 3a Starter culture CHL-N01 at 30°C incubation temperature; 3b Starter culture CHL-N01 at 22°C incubation temperature; 3c Starter culture CHL 44 at 30°C incubation temperature; 3d Starter culture CHL 44 at 22°C incubation temperature. Total colony count, •; *Streptococcus cremoris*, o ; *Streptococcus lactis*, Δ ; aroma bacteria, □.

process is unlikely to cause problems in practice, because the data show quite clearly that this aspect of culture physiology was, at least for these starters, only diminished after several transfers at 30°C.

It was not anticipated that any profound correlation would exist between cell numbers and the production of acidity, but as the same bacteria are also involved in the ripening of cheese, either directly or through the provision of the appropriate conditions within the cheese, changes in the species composition of a starter could be significant.

The behaviour of the two starters, as reflected in the colony counts, is shown in Figure 3, and it is noticeable that temperature of incubation can dramatically alter the balance between, as well as the total numbers of, the various species.

At 30°C, the most obvious effect of sub-culturing is the steady decline in the total counts of the constituent species/strains, and only the strains of *Str. lactis* in CHL44 appear to thrive at the elevated temperature. Nevertheless, the overall trend is clearly deleterious as far as the majority of strains is concerned, and it is probable that the quality of any cheese manufactured with such starters would suffer accordingly.

The position was, however, totally altered when the starters were incubated routinely at 22°C. Thus, the populations of both the aroma bacteria and *Str. lactis* showed remarkable stability for the duration of the experiment, and some of the strains of *Str. cremoris* from culture CHL44 behaved in a similar fashion. Whether the initial decline in the total count of *Str. cremoris* represents a loss of certain strains is a matter for speculation, but it is of note that the strains composing culture CHL-N01 were, in contrast, declining throughout the programme of transfers.

The precise reasons for the population changes would be difficult to elucidate. The relative availability of calcium may be important in respect of certain 'aroma' bacteria (Galesloot and Hassing, 1961), and synergistic/antagonistic interactions may also be involved, but whatever the precise mechanisms, it is clear that:

i) this work confirms the results of Hammer and Babel (1957) that mesophilic starter cultures should be incubated at 21-22°C for optimum activity and strain stability;
ii) the two commercial starter cultures behave entirely differently during routine sub-culturing — a pattern that may well reflect the presence of different strains;

TABLE 1 The effect of previous incubation temperature/time and subculturing on the acid* development by starter cultures during the simulated cheese-making conditions of the vitality test.

Transfer	Starter culture			
	CHL-N01		CHL 44	
	22°C	30°C	22°C	30°C
One	0.71	0.75	0.62	0.73
Two	0.69	0.69	0.65	0.72
Three	0.68	0.67	0.69	0.61
Four	0.62	0.69	0.71	0.53
Five	0.63	0.68	0.58	0.63
Six	0.54	0.69	0.59	0.59
Seven	0.57	0.63	0.59	0.59
Eight	0.53	0.57	0.58	0.63
Nine	0.59	0.69	0.63	0.59
Ten	0.58	0.68	0.59	0.65

* Average of two identical runs after six hours of incubation at 30°C.

iii) the species in certain commercial cultures stabilise rapidly during serial transfers, and in acceptable numbers.

The behaviour of starters under cheesemaking conditions (30°C) showed that, when the cultures were previously grown at 30°C, acid production was fairly stable, as sub-culturing proceeded, and higher than at 22°C (Table 1). While this pattern would suggest that acclimatising the starter to the temperature of cheese-making is advantageous, the data presented in Figures 2 and 3 would appear to suggest otherwise, and the reasons for the variation have yet to be established. One possibility, however, is that the rennet increases the level of assimilable nitrogen in the milk, and that this improved nutritional status compensates for the apparent deficiences that result from the serial transfers at 30°C.

Nevertheless, even allowing for certain anomalies, these findings appear to contradict the normal assertion that complex starter cultures will not withstand routine sub-culturing without deleterious changes in metabolic capacity and/or species composition. If this conclusion is valid, it would imply that creameries in countries with limited access to commercial starter banks need no longer be reticent about resorting to the use of commercial cultures subcultured in their own laboratories. Obviously the correct balance between the species cannot be maintained indefinitely in liquid starters, but given an appropriate choice of initial culture and a high standard of hygiene, then the purchase of commercial cultures could become a monthly rather than daily necessity. Whether this facility would encourage cheesemakers in developing countries to make more use of freeze-dried, commercial starter cultures is another matter, but at least the fear of relying too heavily on imported cultures need no longer be an obstacle.

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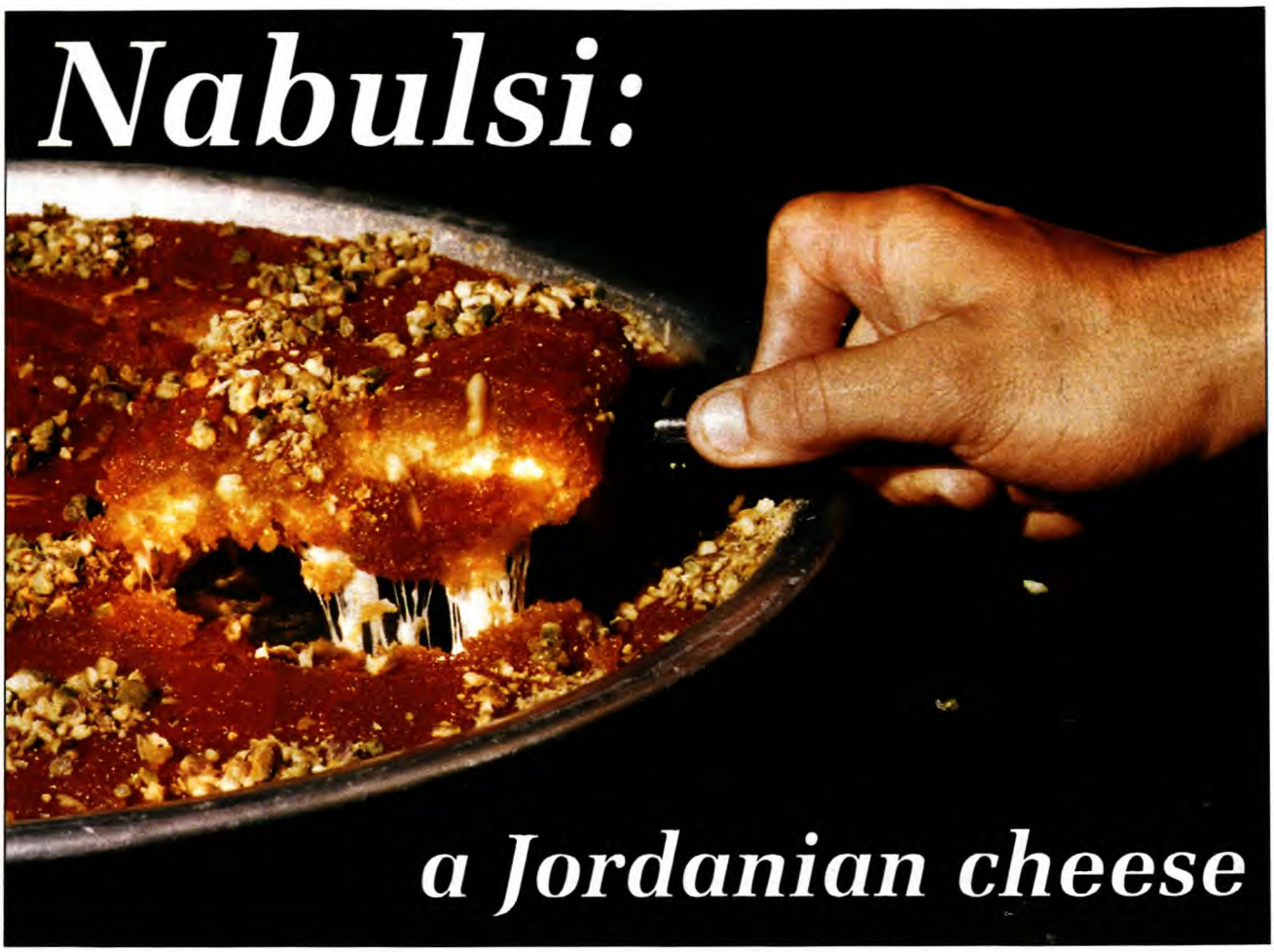
Nabulsi: a Jordanian cheese



A study of the impact of microflora on the sensory properties of Nabulsi cheese, by M S Y Haddadin and R M A Shahin, University of Jordan, Amman and R K Robinson, University of Reading, England

White-brined cheeses have long been popular throughout the Mediterranean and Middle Eastern countries, but storage in brine (4-16%) gives many varieties a salt content that would, in terms of flavour, be unacceptable in Europe or North America.

However, in Jordan, a rather more mild flavoured, white-brined cheese, Nabulsi, is the main cheese produced along traditional lines, and it is widely consumed at breakfast (1) or used in the manufacture of local desserts like Kunafeh (see Figure 1). This latter usage implies that the mild flavour is essential and yet, traditionally, Nabulsi was made from raw sheep's milk and stored for long periods. It is not clear whether a post-production immersion in boiling water reduces the activity of the initial lactic acid microflora and prevents the build-up of excess acidity or harsh flavour but, with recent changes in cheesemaking practice, the question has become more important.

Today, cheese for retail sale has to be produced from pasteurised sheep's milk, or mixtures with cow's or goat's milks. Hence the natural microflora of thermoduric species or casual contaminants have become the main source of ripening activity; few farmers are familiar with the use of starter cultures. For small volumes of milk handled in utensils impregnated with lactic acid bacteria, this haphazard situation may well be acceptable, but as market demand and co-operative ventures grow, so control over the ripening stage will become essential. The nature of any introduced starter culture will be of vital importance, and the aims of this work were to:

- study the development of the natural microflora during the storage of Nabulsi cheese made from pasteurised Awassi sheep's milk; and
- determine the influence of the addition of a starter culture on the properties of the final cheese.

Materials and methods

All the milk came from one herd of Awassi sheep in the El-Yadudah area near Amman, and the milk was collected daily during July for the production of cheese.

Figure 1 (above): Kunafeh is a dessert of Jordanian origin, made by lining a dish with Nabulsi cheese, covering the cheese with apple, brown sugar and nuts, and then baking

The milk was brought to the laboratory in churns and, after warming to 40°C, was filtered through cheese cloth and pasteurised at 63°C for 30 minutes. The milk was then poured into a temperature controlled, stainless steel container equipped with a slow speed agitator, and calcium chloride (0.02% w/v) and 0.02% of standard rennet were added. The milk was held at 32°C for around one hour to coagulate. When starter cultures (1% v/v *Lactococcus lactis* sub-sp *lactis* and *Lact lactis* sub-sp *cremoris* Type 0-180 from Chr Hansen's Laboratorium, Denmark) were employed, the milk was ripened to pH 6.35-6.4 prior to the addition of the calcium chloride and rennet.

Once the coagulum had formed, it was cut into 1cm cubes and natural

Table 1. Typical chemical composition of Nabulsi cheese made from pasteurised sheep's milk with or without the addition of a mesophilic starter culture.
(All figures in g (mg)/100g of mature cheese)

	Natural Microflora	Starter Culture
Fat	28.9	21.5
Protein	19.7	21.4
Moisture	42.5	44.1
Salt	6.4	7.4
Titratable Acidity	0.32	0.46
Tyrosine (mg)	35.2	66.3
Tryptophan (mg)	61.3	128.3

shrinkage of the pieces was allowed to occur over the following 15-20 minutes. The entire contents of the vat were then poured into a mould lined with cheese cloth and, after an initial period of free drainage, the cheese was pressed at 0.4 MPa for 30 minutes followed by 0.8 MPa for two hours.

After the second pressing, the fresh cheese was cut into small 'slabs' (5x4x2cm), and dry salted with coarse salt. After standing at 5°C for 24 hours, the pieces of cheese were transferred to glass jars (see Figure 2) and covered with brine (15%). After two days, the pieces were removed and carefully immersed in boiling brine for 5-7 minutes. Precise control over this stage of the process is not easy, but the production of a visible 'skin' over the surface of the cheese is usually regarded as the end point. The pieces of boiled cheese were returned to the brine (15%) and stored for a month prior to sensory analysis.

Results and discussion

The average casein:fat ratio in the Awassi sheep's milk over the period of the cheesemaking was 0.9, and typical chemical compositions of the finished cheese were taken at the end of one month (2). In addition, an indication of the extent of proteolysis was obtained by estimating the levels of tryosine and tryptophan (3).

The moisture content for the natural Nabulsi cheese was in line with typical market samples, ie 36.1-51.0% (4), as was the salt content (3) which ranged from 5.7-13.3%. However, the impact of starter culture activity was significant ($p<0.05$) with respect to the fat content, and the calculated figure for fat-in-dry-matter (FDM) fell from 50.2% in the non-starter cheese to 38.5% for the cheese with added starter culture. As the Jordanian Standard (1991) for FDM is not less than 40% (5), the effect of the starter culture could cause problems. It was not established why the losses of fat into the whey were higher in the presence of the culture, but the effect of pH on the properties of the casein could have played a part.

The increased acidity in the presence of the lactococci was expected, and the values for tyrosine and tryptophan indicated that the starter bacteria had enhanced the degree of proteolysis (6,7); it is normal for the free amino acids to increase significantly during the storage of white-brined cheeses (8,9).

The figures for tyrosine and tryptophan, together with higher levels of acidity in the cheese with added starter, suggest that the taste panel would detect a considerable difference between samples, and this assumption proved to be correct.

A panel of 60 untrained panellists examined the two contrasted groups of cheeses and a paired preference test was employed to select the best cheese overall (10). The significant ($p<0.05$) preference for the non-starter cheese was apparent with 65% choosing this cheese and 35% preferring the starter culture cheese.

Figure 2 (below): After treatment in boiling brine, the pieces of Nabulsi cheese are stored in brine for one month or longer before consumption

As Nabulsi cheese is often used in desserts, this preference for a mild flavour may not be surprising. However, the finding does suggest that any moves to employ starter cultures on a regular basis will have to involve careful evaluation of the available options.

Both cheeses had total colony counts (Yeastrel Milk Agar at 30° for 72 hours) of 1.6×10^6 at the end of maturation, while the only species of starter bacterium remaining after the boiling stage (*Lact lactis* sub-sp *lactis*) was at a level of <30 cfu/g. This pattern is in marked contrast with most white-brined cheeses (11), and it would appear that the combined influences of heat and salt were sufficient to reduce the viability of both the starter bacteria and many natural contaminants.

Assuming that the perceived harsh flavour was the result of proteolytic enzymes released by autolysis of the starter bacteria and, given that the culture in question is rated as 'medium' with respect to proteolysis (12), the results suggest that cultures for the production of good quality Nabulsi cheese on a routine basis will need careful selection. ■

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A comparison of the chemical, microbiological and sensory characteristics of bovine and ovine Halloumi cheese

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Abstract

Commercial samples of fresh and mature Halloumi cheeses made from ovine or bovine milk were studied in order to establish their chemical, microbiological and sensory characteristics. Significant differences were observed between the two types of Halloumi cheese both when fresh and mature. The free volatile fatty acid (FVFA) content of the cheeses increased with maturation from 483 to 1356 mg kg^{-1} for the ovine product, but lower values (380–1248 mg kg^{-1}) were found in the bovine cheese. During maturation for 40 days, *Enterococcus faecium*, which dominated the microflora of fresh ovine cheese, was replaced by lactobacilli, including a new species, *Lactobacillus cypricasei*, which was not found in the bovine samples. Fewer than 100 cfu g^{-1} lactic acid bacteria (LAB) were present in the fresh bovine cheeses, but a microflora dominated by lactobacilli developed with time. Yeast counts in the mature ovine and bovine cheeses reached 2.3–2.8 $\times 10^5$ cfu g^{-1} and, as some of the yeasts were proteolytic and/or lipolytic, it was assumed that they were having a positive impact of the flavour of the cheeses. The sensory panel distinguished significant differences in texture and flavour between the fresh and mature samples of both ovine and bovine cheeses and, overall, there was a significant preference for the ovine brand.

Keywords: Halloumi cheese; Chemical characteristics; Sensory; Microbiology

1. Introduction

Halloumi is the traditional cheese of Cyprus and, for many years, it has been produced locally from raw ovine milk or mixtures of raw ovine and caprine milk. It is widely popular in Cyprus and other countries of the Eastern Mediterranean but, more recently, the product has gained international acceptance and recognition; total exports of Halloumi cheese from Cyprus have risen to approximately 2500 metric tonnes (Anon., 2000).

The traditional process for making Halloumi cheese is well established (Papademas & Robinson, 1998; Robinson & Wilbey, 1998), and the distinctive feature of the procedure is that blocks of pressed curd (~250 g) are heated (90–95°C) in whey for at least 30 min prior to cooling and dry salting. This fresh product has a characteristic aroma, its texture is elastic and compact with no holes and it is easily sliced. It is popular with consumers and, while large quantities of Halloumi are sold immediately after production, a proportion of the total output is immersed in whey previously used to heat-treat the cheese modified by the addition of 100–120 g L^{-1} NaCl; the blocks are held in this brine for at least 40 days at room temperature. During this maturation, Halloumi changes markedly in taste and texture and the cheese becomes hard, salty and moderately acidic. Although the microbiology and biochemistry of these changes are poorly understood, the sensory properties of mature Halloumi are much appreciated by connoisseurs.

However, to meet the growing demand in the Middle East and Europe, the regulations in Cyprus and elsewhere now allow the more readily available bovine milk to be used in place of the traditional ovine or caprine milk (Cyprus Standard, 1985). The procedure for making Halloumi from pasteurised bovine milk is broadly similar to the traditional process, and a number of studies have found that the gross chemical composition of the end-product is comparable, in most respects, with Halloumi of ovine or caprine origin (Robinson, Haddadin, & Abdullah, 1991; Anifantakis & Kaminarides, 1982, 1984;

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Shaker, Lelievre, Taylor, Anderson, & Gilles, 1987; Economides, Georghiades, & Mavrogenis, 1987).

Nevertheless, there are compositional differences between ovine and bovine milk with respect to their fat and casein contents (Christie, 1995; Jandal, 1996; Alichanidis & Polychroniadou, 1996), and their respective microfloras may well differ as well (Yamani, Al-Nabulsi, Haddadin, & Robinson, 1998). Thus, the ovine and bovine cheeses could each acquire a distinctive microflora(s) that could affect the pattern of maturation of the cheeses in question. For example, yeasts isolated from dairy products have been shown to have both proteolytic and lipolytic activities (Jakobsen & Narvhus, 1996; Welthagen & Vilijoen, 1999), and many species of LAB have the same properties (Litopoulou-Tzanetaki, 1984; Tzanetakis & Litopoulou-Tzanetaki, 1992). Different microfloras could give rise to contrasted patterns of lipolysis and proteolysis, and lead to the emergence of a specific spectrum of those short chain organic acids (C_2–C_5) that can make significant contributions to cheese flavour (Abd El-Salam, 1987; Barbieri et al., 1994; Torres et al., 1995; Moio & Addeo, 1998; Califano & Bevilacqua, 1999; Georgalla, Kandarakis, Kaminarides, & Anifantakis, 1999).

There is good reason to believe that, if the microfloras associated with the production and maturation of bovine Halloumi differ from those linked with ovine cheese, the result would be cheeses of different chemical compositions and, perhaps, sensory properties as well. Consequently, the aim of this investigation was to:

1. Compare the chemical, microbiological and sensory characteristics of two commercial brands of Halloumi cheese manufactured in Cyprus. One brand was produced from local ovine milk, and the other from bovine milk; and
2. Establish whether any of the yeasts or LAB isolated from the cheeses could have an influence on the flavour of the end-product.

2. Materials and methods

Twenty-four shrink-wrapped, retail blocks (250 g) of fresh and mature samples of the two brands of Halloumi cheese were purchased in Cyprus. In each case, retail blocks were selected with identical batch codes/dates of manufacture, and the individual groups of twenty-four blocks were transported to a dairy in Cyprus in an ice box. Eight samples designated for chemical analysis were then frozen at −20°C, while those required for microbiological or sensory analysis were stored at ~4°C. Next day, the blocks were sent in insulated boxes to the authors' laboratory by air-freight for frozen storage (eight samples for chemical analysis) or for chill storage (4°C) prior to microbiological examination within 24 h or sensory analysis within 72 h.

2.1. Microbiological analysis

2.1.1. Examination of the Halloumi cheeses

To establish the total colony counts for LAB and yeasts in the fresh and mature Halloumi cheeses, three blocks of fresh cheese and three blocks of mature cheese — selected at random from the available blocks of each brand — were, in turn, unwrapped in a sterile cabinet and cut into half with a sterile knife. One portion was then shredded with a sterile grater, and a sub-sample (10 g) weighed into a stomacher bag. After blending with Maximum Recovery Diluent (MRD — Oxoid Code No: CM733, Unipath Ltd., Basingstoke, Hants, UK, 90 mL) serial dilutions down to 10^{-6} were made employing 1 mL transfers into 9 mL amounts of sterile MRD. Duplicate aliquots (0.1 mL) from each dilution were spread onto the surfaces of pre-dried plates of MRS Agar (Oxoid Code No: CM361) and, after the liquid had been adsorbed, the plates were incubated aerobically at 30°C for 72 h (McSweeney, Fox, Lucey, Jordan, & Cogan, 1993). Yeasts were isolated and enumerated from the same dilutions on yeast extract dextrose chloramphenicol Agar (IDF, 1990), with the plates incubated at 25°C for 5 days.

After completing the total colony counts, the plates were examined with a hand-lens in order to establish the dominant types of colony. Once the major colony types had been identified on the basis of morphology, colonies of presumptive lactobacilli, Gram-positive cocci and yeasts were confirmed by Gram-staining. Five colonies of each type were then streaked onto plates of MRS agar or yeast extract dextrose agar (minus chloramphenicol), and single colonies were sub-cultured onto slopes of the appropriate medium for subsequent identification.

2.1.2. Identification of lactic acid bacteria

After Gram-staining and checking for catalase activity, isolates of Gram-positive, catalase-negative cocci growing in pairs or chains were identified using the PCR reaction for amplified variable regions of 16S rRNA (Lawson et al., 2001). The same procedure was applied to cultures of Gram-positive, catalase-negative rods.

2.1.3. Identification of yeasts

After recording the morphology of the different types of colony, wet preparations of each type were made in dilute methylene blue, and the characteristic shapes of the cells, type of division and presence/absence of pseudomycelium were noted under ×400 magnification. Further tests, as suggested by Deak and Beuchat (1996), included urease activity, utilisation of nitrate, proteolytic activity and production of lipase or esterase.

2.1.4. Enzymatic profiles

As one of the possible impacts of the microfloras on the cheeses might involve proteolysis or lipolysis, the

enzymatic activities of species of LAB and yeast isolated from fresh or mature samples of Halloumi cheese were tested. The API-ZYM (BioMérieux, Basingstoke, Hants, UK) system was employed according to the manufacturer's instructions, and the range of enzymes being screened (details in Table 3) has been reported to be appropriate for studying cultures of dairy origin (Tzanetakis, Hatzikamari, & Litopoulou-Tzanetaki, 1992; Bintsis, Litopoulou-Tzanetaki, Davies, & Robinson, 2000).

2.2. Chemical composition

Three blocks each of fresh and mature cheese — selected at random from the available blocks of each brand — were removed from the deep-freeze and allowed to thaw overnight. Each block was then shredded with a grater, and appropriate sub-samples removed for analysis. The gross chemical composition of each block was determined in triplicate using the following standard methods: moisture (BS 770: Part 2, 1976), fat (BS 696: Part 2, 1989), total nitrogen (BS 770: Part 5, 1976), salt (BS 770: Part 4, 1989) and pH (BS 770: Part 8, 1976).

2.2.1. Determination of short-chain (C_2–C_5) free volatile fatty acids (FVFA)

The FVFA in three blocks of each type of fresh and mature Halloumi cheese were determined by gas chromatography (GC) according to the method of Fussell and McCalley (1987). The FVFA were extracted from the cheese samples as follows: 10 g of cheese were mixed with 50 mL of distilled water in 100 mL screw-capped bottles, and shaken (60 rpm) for 1 h at room temperature. The mixture was then filtered through a Whatman No. 2 filter paper, and 4 mL of the solution were treated with 1 mL of metaphosphoric acid (200 g L^{-1}). After centrifugation (5 min at room temperature), the solution was stored at 4°C overnight.

The analysis was carried out with a PU 4500 gas chromatograph (Pye Unicam, Cambridge, UK), equipped with a flame ionisation detector (FID) and a 80/120 Carbopack B capillary column (2 m × 2 mm ID glass; Supelco, Bellefonte, USA). The injection and detection temperatures were 250 and 200°C, respectively, while the column temperature was adjusted to 175°C. The flow rate of the carrier gas (H_2) was 30 mL min^{-1}. The area under the peaks was calculated using a SP 4270 integrator (Spectra Physics, San Jose, CA 95134, USA), and the retention indices were compared with a reference mixture. This mixture contained known concentrations of pure short-chain (C_2–C_5) volatile fatty acids, namely ethanoic (acetic), propanoic (propionic), 2-methylpropanoic (isobutyric), butanoic (*n*-butyric), 3-methylbutanoic (isovaleric) and pentanoic (*n*-valeric) (Sigma Ltd., Poole, Dorset) together with lactic acid (Merck Ltd., Poole, Dorset).

2.3. Sensory analysis

The vocabulary used to characterise the samples was generated by ten residents of Cyprus, who were familiar with Halloumi cheese. The individuals were given typical samples of fresh and mature cheese, and were asked to taste them and describe any aspects of flavour and texture that seemed distinctive. Once the individual tasters had provided a list of descriptors, a round-table discussion produced a final vocabulary comprising 12 terms: six covering taste and flavour (salty, bitter, acid, creamy, milky and minty), four for textural characteristics (crumbly, springy, moist, chewy), and two for appearance, i.e. colour (white/yellow) and body (layered).

An informal sensory panel was then arranged at the University of Reading: the membership consisted of ten Cypriot students who ate the product on a regular basis. After a preliminary training session, each panellist was asked to assess two samples of fresh cheese (one ovine and one bovine) and, 15 min later, samples of the corresponding mature products. To obtain the indiviual samples, three retail blocks of each type of cheese were cut into pieces (2.5 × 2.5 × 2.5 cm), and two pieces of fresh cheese (ovine and bovine) or of mature cheese (ovine and bovine) were placed on plain grey plates, covered with 'cling-film', and left for 1 h to reach room temperature (18–20°C). The coded samples were presented to the tasters in individual booths with natural daylight, and the order of tasting was randomised to minimise interference caused by contrast and pattern effects. The rating for each of the 12 attributes was recorded on an anchored line scale (10 cm) with zero equating to an extreme low value, e.g. not salty, not crumbly, and 10 to the extreme opposite (Meilgaard, Civille, & Carr, 1999). In addition, hedonic rating along the same scale was requested for each sample.

2.3.1. Statistical analysis

Analysis of reliance (ANOVA, Microsoft Excel V5.0) was used to determine statistical differences ($P < 0.05$) in the compositions of the cheeses. The results from the gas chromatograph and the sensory panel were analysed using the General Linear Model (Minitab 10.5), and significant differences ($P < 0.05$) between mean values were determined.

3. Results and discussion

3.1. Microbiological differences due to maturation

The total colony counts for LAB and yeasts in the two types of cheese are shown in Table 1. The major difference between the fresh samples of Halloumi cheese was the apparently low number of LAB in the samples made from bovine milk. Clearly the combination of

Table 1
Mean total colony counts of LAB and yeasts (cfu g^{-1}) ($\pm$ standard deviation) in samples of fresh and mature Halloumi cheese made from ovine or bovine milk[a]

	Ovine		Bovine	
	Fresh	Mature	Fresh	Mature
LAB	$2.2 \times 10^4 \pm 1.1 \times 10^4$	$5.6 \times 10^5 \pm 0.3 \times 10^5$	<100	$6.6 \times 10^4 \pm 2.3 \times 10^4$
Yeasts	<100	$2.3 \times 10^5 \pm 0.2 \times 10^5$	<100	$2.8 \times 10^5 \pm 0.5 \times 10^5$

[a]Means of duplicate counts from three individual blocks of cheese.

Table 2
Species of lactic acid bacteria and yeasts isolated from fresh and mature commercial Halloumi cheeses[a]

	Ovine		Bovine	
	Fresh	Mature	Fresh	Mature
LAB	*Enterococcus faecium*	*Lactobacillus cypricasei* *Lactobacillus brevis* *Enterococcus faecium*	ND	*Lactobacillus brevis* *Lactobacillus pentosus* *Lactobacillus plantarum*
Yeasts	*Debaromyces hansenii* *Candida parapsilosis*	*Candida boidinii* *Candida versatilis* *Pichia membanaefaciens*	ND	*Cryptococcus albidus* *Pichia membanaefaciens*

[a]ND = none detected at 10^{-1} dilution.

pasteurisation of the incoming milk and the heat treatment of the cheese had depleted the population severely, whereas the total lactic microflora (Gram-positive cocci and lactobacilli) of the fresh ovine cheeses reached numbers of 10^4 cfu g^{-1}.

As only thermoduric micro-organisms from the original milk are likely to survive the cheesemaking procedure (Poullet, Huertas, Sanchez, Caceres, & Larriba, 1993), it was not unexpected that *Enterococcus faecium* was the most frequent isolate. *Enterococcus* spp. have been isolated in high numbers from many traditional raw milk cheeses (Poullet et al., 1993; Macedo, Maltaka, & Hogg, 1995; Centeno, Menendez, & Rodriguez-Otero, 1996; Freitas et al., 1998) and traditional fermented milk products made with ovine milk (Samolada, Litopoulou-Tzanetaki, Xanthopoulos, & Tzanetakis, 1998). In particular, it may be relevant that some strains of enterococci are proteolytic, and hence, in the present context, could be contributing to the flavour of the Halloumi cheese made from ovine milk (Cogan, 1996; Arizcun, Barcina, & Torre, 1997).

The lactic acid microflora of all the samples of Halloumi apparently, changed with maturation, as Gram-positive, catalase-negative rods were dominant in the mature cheeses; such changes in microflora with maturation were reported also by McSweeney et al. (1993) and Haddadin (1986) who found that lactobacilli were the predominant non-starter LAB in Cheddar and Domiati cheeses, respectively. Several species of the genus *Lactobacillus* were identified from mature samples of Halloumi cheese (Table 2); *Lactobacillus pentosus* and *Lactobacillus plantarum* were isolated only from bovine Halloumi cheese, while *Lactobacillus brevis* was present in both the bovine and ovine cheese samples.

More significant, perhaps, was the isolation, from Halloumi of ovine origin of a unique species of *Lactobacillus* that was given the name of *Lactobacillus cypricasei* (Lawson et al., 2001). This new species was not isolated from any of the cheeses of bovine origin. The frequent presence of *Lb. cypricasei* in the mature ovine cheeses suggests that minor compositional differences between the ovine and bovine milks could have a selective effect on the microflora of the cheese/brine (Anifantakis & Kaminarides, 1981).

Yeasts were isolated from mature bovine and ovine cheeses at levels of 2.8×10^5 and 2.3×10^5 cfu g^{-1}, respectively, but their presence appeared to have no adverse effect on quality, in that no off-flavours, visible colonies or any swelling of packets due to excess CO_2 production (Westall & Filtenborg, 1998) were detected.

Nevertheless, the enzymatic profiles of the yeasts and LAB isolated from fresh and mature samples of Halloumi cheese (Table 3) indicated that the yeast species and *Ent. faecium* possessed esterase and lipase enzymes that could be responsible for lipolytic activity in the cheese matrix. In addition, leucine aminopeptidase was present in all of the isolates, and *Lb. cypricasei* also possessed valine aminopeptidase. These intracellular enzymes can break

Table 3
The enzymatic profiles of selected yeasts and lactic acid bacteria isolated from Halloumi cheese[a]

Species	*Debaromyces hansenii*	*Candida parapsilosis*	*Cryptococcus albidus*	*Candida boidinii*	*Candida versatilis*	*Lactobacillus cypricasei*	*Lactobacillus pentosus*	*Enteroccocus faecium*
Alkaline phosphatase	+	+	+	+	−	+	−	−
Esterase (C4)	+	+	+	+	+	−	−	+
Esterase lipase (C8)	+	+	+	+	+	−	−	+
Lipase	−	−	−	−	−	−	−	+
Leucine aminopeptidase	+	+	+	+	+	+	+	+
Valine aminopeptidase	−	−	−	−	−	+	−	−
Acid phosphatase	+	+	+	+	+	V	−	+
Phosphoamidase	+	+	−	+	−	V	−	+
β-galactosidase	−	−	−	−	−	+	+	−
N-acetyl-β-glucosaminidase	−	−	−	−	−	V	+	−
α-glucosidase	+	+	−	−	−	+	+	−

[a](+) positive; (−) negative; V = variable.

Table 4
Mean chemical composition of fresh and mature commercial Halloumi cheeses[a]

Constituents ($g\,kg^{-1}$)	Fresh		Mature	
	Ovine	Bovine	Ovine	Bovine
Moisture	474.6 ± 25.2 NS	466.3 ± 2.4 NS	349.4 ± 5.1 NS	353.0 ± 9.7NS
Fat	260.8 ± 5.8[b]	243.6 ± 13.8[c]	318.9 ± 12.3[d]	285.8 ± 26.9[e]
TN	31.7 ± 1.8 NS	34.9 ± 2.2 NS	35.8 ± 0.9[d]	44.6 ± 4.6[e]
Protein	202.2 ± 11.5 NS	222.7 ± 14.0NS	228.4 ± 5.7[d]	284.5 ± 29.3[e]
Salt	27.4 ± 5.3 NS	29.7 ± 3.0NS	38.0 ± 0.9[d]	40.4 ± 4.6[e]
PH	5.9 ± 0.1[b]	6.2 ± 0.1[c]	4.6 ± 0.1NS	4.5 ± 0.1NS

[a]Means of three cheese blocks analysed in triplicate ± SD.
[b–c]Superscripts indicates significant differences ($p < 0.05$) between the samples of fresh (b,c) and mature (d,e) cheeses, respectively.
NS = no significant difference.
TN = total nitrogen.

down peptides to amino acids, and hence significantly enhance the flavour of a cheese (Mulholland, 1997); the amino acids can also serve as substrates for the generation of compounds, such as free volatile fatty acids, that may contribute flavour notes like 'cheesy' or 'sweaty' to a cheese (Moio & Addeo, 1998; Torres et al., 1995).

3.2. Chemical characteristics

The fat and pH values of the fresh samples of Halloumi cheese were significantly different, but the moisture, total nitrogen and salt contents were similar (see Table 4). The different fat contents can be attributed to the different types of milk used for the production of the cheeses, while the slightly higher acidity of the ovine cheese suggests that some microbial activity may have occurred during storage of the ovine milk.

The mature samples did not differ in pH value or moisture content, but there were significant differences with respect to fat, salt and total nitrogen (TN) (see Table 4). Thus, the level of TN in the ovine product was $35.8\,g\,kg^{-1}$ compared to $44.6\,g\,kg^{-1}$ for the bovine cheese, while the fat contents were 318.9 and $285.8\,g\,kg^{-1}$ for the ovine and bovine products, respectively. The changes in fat content reflect the overall increase in total solids, but the change in TN suggests that proteolysis was more active in the ovine product so that, as a consequence, more soluble degradation products were lost into the brine (Abd El-Salam, Alichanidis, & Zerfiridis, 1993). The increase in salt content during maturation indicates migration from the brine.

The fermentation of lactose by non-starter LAB was probably responsible for the increase in lactic acid (see Table 5) in mature Halloumi which, in turn, caused a drop in pH to near the isoelectric point of the caseins. As a result, the colloidal calcium was partially solubilised, causing shrinkage of the cheese matrix and the exudation of serum (Abd El-Salam, 1987). The dramatic drop in moisture content during the maturation of Halloumi cheese was probably due to this exudation of serum, as well as a general migration of water into the strong brine.

The FVFA detected were acetic (ethanoic), propionic (propanoic), isobutyric (2-methylpropanoic), butyric

Table 5
Mean free volatile fatty acid (FVFA) (mg kg^{-1}) contents of fresh and mature commercial Halloumi cheeses ± standard deviations[a]

	Fresh		Mature		Statistical analysis		
	Ovine	Bovine	Ovine	Bovine	Milk (M)	Age (A)	M•A
	Free volatile fatty acids (mg kg^{-1})						
Ethanoic	189.19 ± 77.69	162.50 ± 33.95	1093.50 ± 55.35	1079.75 ± 77.53	NS	[b]	NS
Propanoic	107.81 ± 16.3	129.76 ± 15.68	60.94 ± 7.86	72.50 ± 7.14	NS	[c]	NS
2-methylpropanoic	42.45 ± 13.60	12.5 ± 0	39.58 ± 9.55	16.67 ± 7.22	[c]	NS	NS
Butanoic	28.13 ± 13.26	21.88 ± 4.42	36.84 ± 18.50	22.92 ± 3.61	NS	NS	NS
3-methylbutanoic	68.81 ± 16.8	52.95 ± 17.92	31.25 ± 8.84	26.53 ± 15.58	NS	NS	NS
Pentanoic	46.88 ± 13.26	0	88.98 ± 19.73	25.00 ± 0.00	[b]	[b]	NS
Total	483.27	379.59	1356.25	1248.03	NA	NA	NA
Lactic acid	5160.00 ± 393.16	2050.21 ± 89.65	13,925.00 ± 331.38	11,137.19 ± 140.73	[b]	[b]	NS

[a]Means of three cheese blocks analysed in duplicate.
NS = no significant difference.
NA = not applicable.
[b]$p < 0.001$.
[c]$p < 0.01$.

(butanoic), isovaleric (3-methylbutanoic) and valeric (pentanoic). The results shown in Table 5 indicate that there were differences between samples depending on the age and the source of the cheese. The ovine Halloumi cheese had higher overall concentrations of FVFA in both the fresh and the mature samples (483 and 1356 mg kg^{-1}, respectively) compared with the bovine cheese which contained 380 mg kg^{-1} when fresh and 1248 mg kg^{-1} after maturation. The increases in FVFA during maturation were primarily due to the sharp increase in ethanoic acid and, as the flavour threshold for detection by humans can be as low as 22 mg kg^{-1} (Brennand, Ha, & Lindsay, 1989), this increase could well affect the flavour of the cheese. The increased concentration of pentanoic acid from 47 to 89 mg kg^{-1} for the ovine samples as against 25 mg kg^{-1} in the mature bovine samples could again be important, for pentanoic acid can impart a 'sheep-like' flavour note to dairy products.

3.3. Sensory analysis

Although the number of panellists was too small and the extent of their training too limited for the results to be anything but tentative, it was clear that the major differences between the ovine and bovine samples of the fresh cheese were related to texture. In particular, the ovine cheese was significantly more 'springy' and 'chewy' than the bovine brand; this contrast may have been a major determinant of the hedonic rating as well, since there was a significant preference for the ovine cheese. The failure of the panel to identify statistically significant differences in flavour was somewhat unexpected, as the total level of flavour-related FVFA in the ovine sample was higher than in the bovine cheese.

At maturity, flavour notes like salty, bitter or acidic increased significantly in intensity, and the structure of the cheese changed dramatically as well. In particular, the mature cheese was much less 'springy' than the fresh sample and less 'moist', while proteolysis and other reactions caused the layered structure (body) of the cheese to become less obvious. Again there was an overall preference for the cheese made with ovine milk, even though no significant differences for specific flavour attributes emerged.

4. Conclusion

While Halloumi cheese made from bovine milk enjoys an extensive market, it does appear that, when a direct comparison is possible, consumers would prefer the traditional ovine cheese. Compositional differences with respect to the concentrations of free fatty acids or amino acids, for example, may explain, at least in part, why the origin of a cheese can be detected, and contrasted microfloras (ovine *versus* bovine) of the milk and storage brine may be the principal source(s) of variability. Whether the microbiological differences between the cheeses observed in this study are widespread and/or consistent remains to be established, but there are indications that there is indeed a characteristic microflora associated with ovine Halloumi produced in Cyprus.

The role of other factors, such as the feeding habits of sheep and cows, could be relevant as well; in a preliminary study (unpublished data) the terpenes present in Halloumi made in Cyprus from ovine milk were different from those in bovine Halloumi. Whether or not the concentrations are sufficient to influence consumer reactions needs to be assessed.

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Lactobacillus cypricasei sp. nov., isolated from Halloumi cheese

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Four strains of a hitherto unknown bacterium isolated from Halloumi cheese were compared by using phenotypic and phylogenetic studies. Comparative 16S rRNA gene sequencing demonstrated that the strains were identical to each other and represent a new subline within the genus *Lactobacillus*. The unknown bacterium was readily distinguished from other described Gram-positive catalase-negative taxa by means of biochemical tests and electrophoretic analysis of whole-cell proteins. On the basis of phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium be classified as *Lactobacillus cypricasei* sp. nov. The type strain of *L. cypricasei* is CCUG 42961^T (= CIP 106393^T).

Keywords: *Lactobacillus cypricasei*, Halloumi cheese, 16S rRNA, taxonomy, phylogeny

INTRODUCTION

The lactic acid bacteria possess a large number of metabolic properties that are responsible for their successful use as starter cultures in the commercial production of fermented dairy, meat and vegetable products and beverages (Hammes *et al.*, 1992: Stiles & Holzapfel, 1997; Wibowo *et al.*, 1985). The genus *Lactobacillus* represents the largest group of rod-shaped organisms within the lactic acid bacteria: currently, over 60 species are recognized. Some members of this group of organisms are important in the generation of particular flavours and in other ripening processes associated with specific cheeses (Elsoda, 1993; Elortondo *et al.*, 1998; Fitzsimons *et al.*, 1999; Hong *et al.*, 1998). Halloumi cheese originated in Cyprus, but its appeal has spread worldwide. It is semi-hard, elastic, has no obvious skin/rind, has a close texture with no holes and is easily sliced. The colour varies from white (when a mixture of ovine/caprine milk is used) to yellowish (when bovine milk is the main ingredient). A feature of the production method of this particular cheese is that no starter cultures are used; rather, the flavour and texture depend solely on the indigenous microflora of the milk (Papademas & Robinson, 1998). During the course of a study to determine the microflora of Halloumi cheese, organisms corresponding to *Lactobacillus brevis*, *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Enterococcus faecium* were recovered. In addition, a hitherto unknown *Lactobacillus*-like bacterium was isolated. In this article, we report the characteristics of this unknown lactic acid bacterium and the results of a polyphasic taxonomic study. On the basis of the results of this study, a new *Lactobacillus* species, *Lactobacillus cypricasei* sp. nov., is described.

Abbreviation: MRD, maximum recovery diluent.

The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 42961^T is AJ251560.

METHODS

Bacterial strains and cultivation. Strains LMK1, LMK2 and LMK3 were isolated from Halloumi cheese in which ovine milk from a dairy located in the province of Paphos, Cyprus was used as the major ingredient. Strain LMD2 was isolated from Halloumi cheese made from ovine milk from a dairy located in the province of Nicosia, Cyprus. The cheese-making processes were as described previously (Papademas & Robinson, 1998). Two blocks of cheese of each type were unwrapped and cut in half. One portion was then shredded with a grater and 10 g weighed into a stomacher bag. The cheese was then blended with 90 ml maximum recovery diluent (MRD; catalogue no. CM 733; Oxoid, Unipath) and serial dilutions made to 10^6 using MRD. Duplicate aliquots from each dilution were put onto MRS agar (De Man *et al.*, 1960) and incubated aerobically at 37 °C for 72 h. Total colony counts were taken and the plates examined with a hand-lens in order to establish the dominant colony types. Five colonies each of the predominant types were taken and subcultured on MRS agar. Strains LMK1, LMK2, LMK3

and LMD2 have been deposited in the Culture Collection of the University of Göteborg (CCUG) under accession numbers 42959, 42960, 42961^T and 42962, respectively.

Biochemical characterization. The strains were biochemically characterized by using the API rapid ID32 Strep, API ZYM and API CH50 systems according to the instructions of the manufacturer (API bioMérieux). The type strains and other reference strains used in the investigation were all maintained by the CCUG, Sweden. All tests were performed in duplicate.

SDS-PAGE of whole-cell proteins. To assess the overall phenotypic resemblance of the new isolate and reference species, a comparative analysis of whole-cell protein profiles by SDS-PAGE was performed. PAGE analysis of whole-cell proteins was carried out as described by Pot *et al.* (1994) and Vandamme *et al.* (1998). For densitometric analysis, normalization and interpretation of protein patterns, the GCW version 3.0 software package (Applied Maths) was used. For each pair of traces, the similarity was expressed by the Pearson product moment correlation coefficient, converted (for convenience) to a percentage similarity.

Determination of 16S rRNA gene sequences and phylogenetic analysis. Phylogenetic analysis was performed by using comparative 16S rRNA gene sequence analyses. A large fragment of the 16S rRNA gene (corresponding to positions 30 to 1521 of the *Escherichia coli* 16S rRNA gene) was amplified by a PCR using conserved primers close to the 3′- and 5′-ends of the gene. The PCR products were directly sequenced using a *Taq* DyeDeoxy terminator cycle-sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolate were determined by performing database searches. These sequences and those of other known related strains were retrieved from the EMBL or Ribosomal Database Project databases and aligned with the newly determined sequences by using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and approximately 100 bases at the 5′-end of the rRNA were omitted from further analyses because of alignment ambiguities. Pairwise evolutionary distances were computed from a continuous stretch of 1320 bases by using the correction of Jukes & Cantor (1969). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

RESULTS AND DISCUSSION

All four strains consisted of Gram-positive, non-spore-forming, rod-shaped organisms, which occurred as single cells, pairs or short chains. The mean dimensions of the rods were 0·6–0·8 μm (width) and 3–5 μm (length) and they had rounded ends (Fig. 1). The isolates were facultatively anaerobic and catalase- and oxidase-negative. No growth was observed at 15 or 45 °C in MRS broth after 48 h incubation. With commercial API systems, acid was produced from arbutin, cellobiose, galactose, glucose, D-fructose, D-mannose and salicin but not from D-arabinose, adonitol, D-arabitol, cyclodextrin, dulcitol, erythritol, D-fucose, L-fucose, glycogen, inositol, mannitol, melibiose, melezitose, methyl β-D-glucopyranoside, pullulan, sorbitol, tagatose, D-raffinose or D-xylose. Acid production from L-arabinose, lactose, sucrose and trehalose was found to be variable. Results for acid production from ribose, maltose and *N*-acetyl-β-glucosamine differed according to the different API systems employed. With the API CH50 kit, acid was produced with these substrates, but, with the API rapid ID32 Strep system, these substrates were not fermented. Gas was not produced from glucose. The isolates gave positive reactions for arginine dihydrolase, β-galactosidase, β-galacturonidase, α-glucosidase, β-glucosidase, leucine arylamidase, pyroglutamic acid arylamidase (weak reaction) and valine arylamidase. Negative reactions were observed for alanine phenylalanine proline arylamidase, alkaline phosphatase, chymotrypsin, esterase C-4, ester lipase C8, α-fucosidase, β-glucuronidase, glycine-tryptophan arylamidase, lipase C14, β-mannosidase, trypsin and urease. Variable reactions were obtained for acid phosphatase, cystine arylamidase, α-galactosidase, α-mannosidase and phosphoamidase. All of the isolates hydrolysed aesculin but not hippurate and gave positive VP test results.

Fig. 1. Photomicrograph of *Lactobacillus cypricasei* sp. nov., after 24 h growth in maximum recovery diluent broth, showing the typical rod-shaped morphology.

In terms of their cultural and biochemical characteristics, the isolates from Halloumi cheese resembled the genus *Lactobacillus* but did not correspond to any recognized species. To investigate the overall phenotypic resemblance of the unknown cheese isolates to each other and to species of the genus *Lactobacillus*, whole-cell protein profiles were determined. A numerical analysis of the PAGE protein patterns of the unknown *Lactobacillus*-like strains together with some reference species of *Lactobacillus* are shown in Fig. 2. The four cheese isolates clustered together and formed a distinct group with a correlation level of 85%. *L. brevis* CCUG 30670^T was the species nearest to the unknown isolates, joining the latter group at a correlation of approximately 65% (Fig. 2). Other *Lactobacillus* species were more distantly related. The PAGE

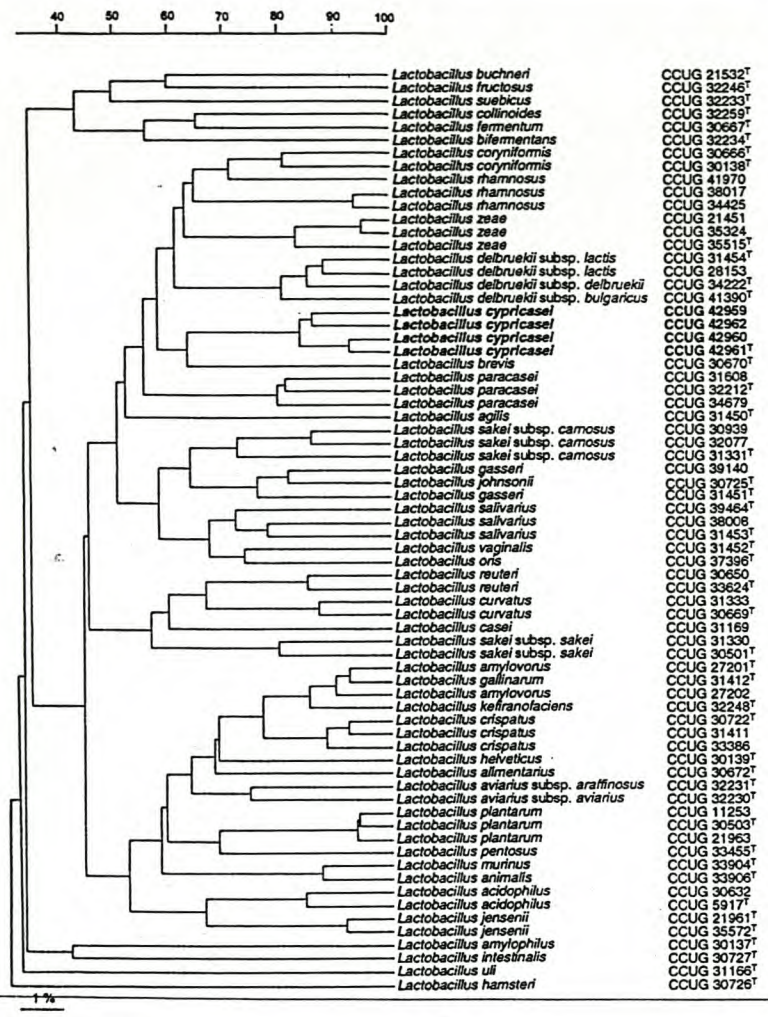

Fig. 2. Similarity dendrogram based on whole-cell protein patterns of *L. cypricasei* sp. nov. and related species. Patterns between 20 and 120 kDa were used in the construction of the dendrogram. Levels of correlation are expressed as percentages of similarity for convenience.

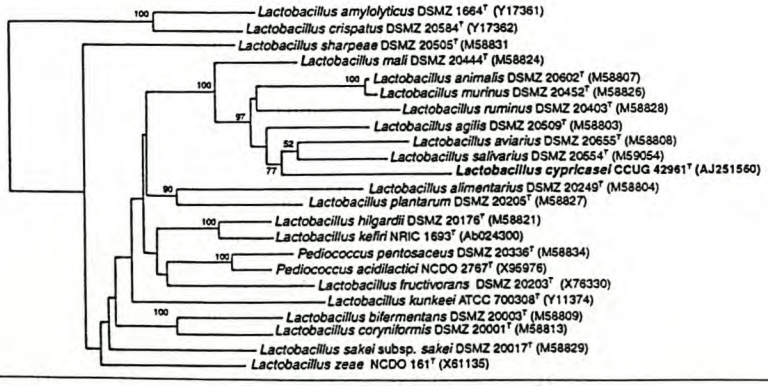

Fig. 3. Unrooted tree showing the phylogenetic relationships of *L. cypricasei* sp. nov. and some related Gram-positive bacteria. The tree constructed using the neighbour-joining method was based on a comparison of approximately 1327 nucleotides. Bootstrap values, expressed as percentages of 500 replications, are given at the branching points. Bar, 1% sequence divergence.

results demonstrated that the four lactic acid strains represent a phenotypically homogeneous group of organisms and are separate from the other *Lactobacillus* species investigated.

To ascertain the phylogenetic relationships of the unknown isolates, their 16S rRNA genes were sequenced and subjected to a comparative analysis. The almost complete gene sequences (> 1450 nucleotides) of the four strains were determined and pairwise analysis showed them to be identical (100% sequence similarity), thereby demonstrating the high level of genealogical relatedness of the isolates. Sequence database searches showed that the unknown bacterium

Table 1. Characteristics that differentiate *Lactobacillus cypricasei* sp. nov. from some of its nearest phylogenetic relatives

Biochemical tests were performed using API rapid ID32 Strep, API ZYM and API CH50 systems (API bioMérieux). v, Variable.

Test	*L. agilis*	*L. animalis*	*L. aviarius*	*L. cypricasei*	*L. murinus*	*L. ruminis*	*L. salivarius*
Production of acid from:							
Galactose	+	+	−	+	+	+	+
Mannitol	+	−	−	−	−	−	+
D-Ribose	+	+	−	v	+	−	−
D-Raffinose	+	+	−	−	v	+	+
Sorbitol	−	−	−	−	−	−	+
Production of:							
Alanine phenylalanine proline arylamidase	+	+	+	−	+	+	+
N-Acetyl β-glucosaminidase	+	+	−	v	+	+	+
Arginine dihydrolase	−	−	−	+	−	−	−
α-Galactosidase	+	+	v	v	+	+	+

was most closely related to species of rRNA group II (*Lactobacillus casei/Pediococcus* group) of the genus *Lactobacillus* (see Collins *et al.*, 1991). The results of neighbour-joining analysis are shown in Fig. 3 and confirmed the association between the unknown bacterium from Halloumi cheese (as exemplified by strain CCUG 42961^T) and rRNA group II of the genus *Lactobacillus*. The unknown bacterium formed a distinct subline within a cluster of species which includes *Lactobacillus agilis*, *Lactobacillus aviarius*, *Lactobacillus animalis*, *Lactobacillus murinus*, *Lactobacillus ruminus* and *Lactobacillus salivarius* (92–93·3% sequence similarity) and was supported by a bootstrap value of 97%. The highest levels of sequence relatedness were found with *L. aviarius* (originally isolated from the intestines and faeces of chickens; Fujisawa *et al.*, 1984) and *L. salivarius* (found in the intestinal tracts of humans and animals; Rogosa *et al.*, 1953), each of which showed a sequence divergence value of 7% with respect to the unknown bacterium. It is clear from the investigation that the *Lactobacillus*-like strains recovered from Halloumi cheese represent a homogeneous group of organisms that are phenotypically distinct from currently recognized members of the genus. Phylogenetically, the unknown bacteria form a distinct subline within the genus; the 7% (or more) sequence divergence values observed with other lactobacilli unequivocally demonstrate that the isolates from cheese warrant classification as a new species of the genus *Lactobacillus*, for which the name *L. cypricasei* sp. nov. is proposed. The characteristics that are useful in distinguishing *L. cypricasei* from its closest phylogenetic relatives are shown in Table 1.

Description of *Lactobacillus cypricasei* sp. nov.

Lactobacillus cypricasei (cy.pri.ca'se.i. N.L. masc. gen. n. *cypricasei* of cheese from Cyprus, referring to the original isolation source).

The cells are Gram-positive, non-spore-forming, rod-shaped and occur as single cells, pairs or short chains. The rods are 0·6–0·8 μm wide × 3–5 μm long, with rounded ends. When grown on MRS agar, colonies are small, entire and cream-coloured. Facultatively anaerobic. Catalase- and oxidase-negative. No growth is observed at 15 or 45 °C in MRS broth after 48 h incubation. The optimum pH range is 5·5–7·5. Homofermentative. No gas is produced from glucose metabolism. Acid is produced from arbutin, cellobiose, galactose, glucose, D-fructose, D-mannose and salicin but not from D-arabinose, D-arabitol, adonitol, cyclodextrin, dulcitol, erythritol, D-fucose, L-fucose, glycogen, inositol, mannitol, melibiose, melezitose, methyl β-D-glucopyranoside, pullulan, sorbitol, tagatose, D-raffinose, xylitol or D-xylose. Acidification of L-arabinose, lactose, maltose, sucrose and trehalose is variable. Positive reactions are obtained for arginine dihydrolase, β-galactosidase, β-galacturonidase, α-glucosidase, β-glucosidase, leucine arylamidase, pyroglutamic acid arylamidase (weak reaction) and valine arylamidase. Negative reactions are obtained for alanine phenylalanine proline arylamidase, alkaline phosphatase, chymotrypsin, esterase C-4, ester lipase C8, α-fucosidase, β-glucuronidase, glycine-tryptophan arylamidase, lipase C14, β-mannosidase, trypsin and urease. Variable reactions are obtained for acid phosphatase, *N*-acetyl-β-glucosaminidase, cystine arylamidase, α-galactosidase, α-mannosidase and phosphoamidase. Aesculin is hydrolysed but hippurate is not hydrolysed. Acetoin is produced. Isolated from Halloumi cheese, a cheese prevalent in Cyprus. The type strain is CCUG 42961^T (= CIP 106393^T).

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ORIGINAL RESEARCH

The sensory characteristics of different types of halloumi cheese as perceived by tasters of different ages

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Three different types of halloumi cheese were assessed immediately after production and after 40 days in brine (~12% NaCl) by two taste panels, using a customized evaluation form based upon a quantitative descriptive analysis schedule. The average ages of the panels were 23 and 45, respectively. The younger panel was more conscious of the overall difference between the fresh and mature cheeses, and specifically with respect to the comparative chewiness and layered appearance of the young cheese. By contrast, the older panel was more sensitive to the salty and minty attributes of the cheeses, as well as differences in crumbliness and colour. Increasing age and/or familiarity with the product did not appear to change the tasters' perceptions of the cheeses to the extent that might have been anticipated.

Keywords Age of panellists, Bovine milk, Halloumi cheese, Ovine milk, Sensory properties.

INTRODUCTION

Halloumi is the traditional cheese of Cyprus and it has been widely popular, both in Cyprus and in other countries of the eastern Mediterranean, for many years. The accepted process for making halloumi cheese involves the coagulation of raw ovine milk or mixed ovine and caprine milks, and the transformation of the gel into a semihard cheese in the manner of feta cheese.[1] The important difference arises in that blocks of this cheese ($10 \times 10 \times 3$ cm) are then heated at 92–95°C in whey extracted during the draining of the curd. On removal from the hot brine, the blocks of warm cheese are folded in half, and sprinkled with a mixture of coarse salt and finely chopped mint (*Mentha viridis*). The fresh product has a characteristic aroma, its texture is elastic and compact with no holes, and it is easily sliced. During maturation, halloumi changes markedly in taste and texture, and it becomes hard, salty and moderately acidic.[2,3] The latter sensory properties of mature halloumi are much appreciated by connoisseurs of this particular type of cheese.

More recently, halloumi has gained international acceptance and recognition and, over the last four years, total exports from Cyprus have risen to approximately 2500 metric tonnes.[4] As a consequence of the increasing demand, local levels of production have had to be raised, and the regulations in Cyprus now permit the manufacture of halloumi cheese from bovine milk. Some consumers have expressed doubts about the ability of this new 'industrial' halloumi to match the organoleptic quality of the caprine/ovine product, but a preliminary evaluation of some commercial samples made from caprine/ovine or bovine milks suggested that reactions to the cheeses varied with the age of the consumer. This point was, in a rather different context, exposed by Lawlor and Delahunty,[5] who found that the appreciation of contrasted varieties of cheese ranging from mild through to highly flavoured types depended upon the average age of the taste panel.

Consequently, it was concluded that any serious evaluation of the sensory properties of the different types of halloumi cheese would have to take the age of the panellists into consideration, and hence the specific aims of this study were to: (i) select three popular brands of halloumi cheese—two made from ovine/caprine milks by traditional, small-scale processes (caprine/ovine 1 and 2), and one manufactured on an industrial scale from bovine milk (bovine); (ii) analyse the cheeses for the major components, e.g. moisture, fat and salt, that might affect the sensory properties of the cheeses; and, (iii) expose the cheeses to two taste panels of Cypriot nationals familiar with halloumi cheese, and to determine to what extent the results of the analysis might vary with the average age of the participants on a particular panel.

MATERIALS AND METHODS

Shrink-wrapped retail blocks (250 g) of fresh and mature samples of the three brands of halloumi cheese were purchased in Cyprus, and replicate blocks were selected to have identical batch codes/dates of manufacture. The blocks were transported to the laboratory in an ice box, and stored at 4°C for a maximum of 4 days before tasting or transport to

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Reading, UK, for further sensory analysis. The samples required for chemical analysis were stored at −20°C before being sent by air-freight to Reading in insulated boxes.

According to Kaminarides *et al.*,[6] the response of tasters to halloumi cheese can depend on the relative proportions of caprine and ovine milk, but as the brands selected for this study were of commercial origin, it had to be assumed that the ratio between the milks was acceptable.

Chemical analysis

The gross chemical composition of the cheese samples was determined using standard methods of analysis: moisture,[7,8] total nitrogen[9,10] and pH.[11] The crude protein contents were calculated by multiplying the figure for total nitrogen by 6.38.

Sensory analysis

Whereas most adults in Europe are familiar with the texture and flavour of semihard cheeses such as Edam, halloumi provides a first-time consumer with a completely new experience with respect to mouth-feel and flavour. Consequently, it was decided to restrict the panels to tasters of Cypriot or Greek origin, and the vocabulary used to characterize the samples was generated during an informal discussion with 10 residents of Cyprus who ate halloumi cheese on a regular basis. Each individual was given blocks (2.5 × 2.5 cm) of typical samples of fresh and mature cheese, and was asked to examine each block in turn and describe: (i) colour and body structure (layered or smooth in cross-section); (ii) physical characteristics, e.g. springiness, crumbliness and moistness, as revealed by the fingers and/or chewing; and (iii) taste and flavour, and any aspects of flavour and texture that seemed distinctive. Once the individual tasters had provided a list of descriptors, a round-table discussion produced a final vocabulary comprising 12 terms, covering taste and flavour (salty, bitter, acid, creamy, milky and minty), textural characteristics (crumbly, springy, moist, chewy), colour (white/yellow) and body structure.

Formal sensory panels of tasters familiar with the product were arranged both at the University of Reading and at a local dairy in Cyprus. The panel in Reading consisted of male and female students with an average age of 23 (± 2 standard deviation), while, in Cyprus, the participants (male) ranged in age from 35 to 70—average age 45 ± 5. After a preliminary training session, each panellist had to assess three samples of fresh cheese along with the corresponding mature products; an interval of 15 min was provided between the tasting of the fresh and mature samples. The coded samples were presented in random order to minimize interference caused by contrast and pattern effects, and the rating for each of the 12 attributes was recorded on an anchored line scale (10 cm), with zero equating to the extreme low value (e.g. not salty, not crumbly), and 10 to the extreme opposite.[12]

Although the use of a quantitative descriptive analysis approach with untrained panellists is not advisable,[12] it was used on this occasion because: (i) all the tasters were familiar with halloumi cheese and its characteristics; and (ii) it seems the most appropriate method for obtaining a 'product description' of halloumi cheese, together with an indication as to which individual attributes of the cheese were most detectable.

Statistical analysis

The data were analysed using the General Linear Model (Minitab 10.5; Minitab Inc., PA, USA) and a factorial model was fitted with two *f* components, i.e. manufacturing processes (two levels) and age (two levels). Significant differences between the mean scores for each attribute ($P < 0.05$) were tested.

RESULTS AND DISCUSSION

The results of the chemical analyses are shown in Table 1, and it was notable that the fresh cheeses differed with respect to fat, protein and acidity/pH according to origin/type of milk. The lower fat content of the bovine cheese suggests that standardization of the milk in the larger plant is under

Table 1 The gross chemical composition of commercial halloumi cheeses. All figures (g/100 g) of cheese as consumed are means of six determinations (three samples tested in duplicate) ± SD

Sample	*Moisture*	*Fat*	*Protein*	*Salt*	*pH*
Fresh cheese					
Caprine/ovine (1)	47.5 ± 2.5	26.1 ± 0.6	20.2	2.7 ± 0.5	5.9 ± 0.1
Caprine/ovine (2)	48.7 ± 1.2	24.8 ± 1.2	18.6	3.0 ± 0.6	6.0 ± 0.1
Bovine	46.6 ± 0.2	24.4 ± 1.4	22.3	3.0 ± 0.3	6.2 ± 0.1
Mature cheese					
Caprine/ovine (1)	34.9 ± 0.5	31.9 ± 1.2	22.8	3.8 ± 0.1	4.9 ± 0.2
Caprine/ovine (2)	32.3 ± 0.3	31.4 ± 1.5	25.5	5.7 ± 0.1	4.9 ± 0.5
Bovine	35.3 ± 1.0	28.6 ± 2.7	28.5	4.0 ± 0.2	4.5 ± 0.1

Table 2 Mean scores (%) for selected attributes of halloumi cheese given by 10 panellists of average age 21

	Fresh halloumi cheeses			*Mature halloumi cheeses*			*Statistical analysis*	
Attributes	*Caprine/ Ovine 1*	*Caprine/ Ovine 2*	*Bovine*	*Caprine/ Ovine 1*	*Caprine/ Ovine 2*	*Bovine*	*Manufacturing procedure*	*Age of the cheese*
Texture								
Springy	64.1	63.5	44.9	23.9	7.8	11.9	*	***
Moist	64.6	39.8	43.6	23.1	12.4	24.3	*	***
Chewy	69.2	62.1	53.6	46.0	25.0	32.4	NS	***
Crumbly	57.3	52.6	49.6	54.5	59.8	64.3	NS	NS
Taste and flavour								
Salty	52.2	21.9	38.9	60.6	82.5	70.5	NS	***
Bitter	8.9	8.0	21.4	45.9	53.4	57.4	NS	***
Acidity	13.5	6.8	18.3	41.2	56.2	54.5	NS	***
Creamy	46.5	40.6	31.1	21.9	19.0	34.4	NS	*
Milky	58.7	53.7	45.3	32.9	32.0	37.1	NS	**
Minty	33.9	55.9	53.5	38.0	39.2	34.5	NS	NS
Overall flavour and preference								
Hedonic	78.4	61.1	52.4	47.9	33.0	32.3	*	***
Flavour intensity	66.2	59.2	53.9	56.9	60.3	52.5	NS	NS
Appearance								
Colour	49.7	63.4	43.2	40.2	60.4	54.7	NS	NS
Body	75.0	43.0	62.8	41.3	45.9	41.8	*	***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

NS = no significant difference

stricter control than in the factory manufacturing caprine/ovine (1), but the lower pH of the same caprine/ovine cheese may well be linked to the fact that the original milk had a more active and varied microflora of nonstarter lactic acid bacteria (data not shown). As the metabolic activities of the nonstarter lactic acid bacteria often have a beneficial impact on cheese flavour,[13] this contrast between the products could be important.

After maturation for 40 days in brine, the moisture and salt contents were markedly different (perhaps as a result of different concentrations of NaCl in the holding brines), but the pH values tended to become similar as a result of microbial activity during storage. The precise reason for the high salt content of the mature caprine/ovine (2) was not investigated, but the low moisture content of the same cheese suggests that the storage brine had too high a level of NaCl.

The results of the taste panel of students are shown in Table 2, and it is clear that there were some differences between the fresh cheeses of caprine/ovine origin with respect to physical characteristics. In particular, the caprine/ovine (1) cheese was perceived as feeling more moist—even though the moisture contents of both caprine/ovine cheeses (Table 1) were similar, and the layered structure was noted as a significant feature; the bovine cheese was recorded as being less springy than its ovine counterparts. None of the attributes for taste and flavour were recorded as significantly different between the cheeses, but there was a strong overall preference for the cheeses of caprine/ovine origin, and especially for caprine/ovine (1).

At maturity, there was a marked decline in overall acceptability, as tastes such as salty, bitter and acid increased significantly in intensity (see Table 2), and these latter responses reflect the chemical changes shown in Table 1. Curiously, while the panel was aware of an increase in saltiness/acidity with maturation, these differences did not seem to contribute to an overall increase in flavour intensity. Significant changes in the springiness and chewiness were recorded, and the loss of whey into the brine (see Table 1) had a significant effect on the perceived moistness of the cheese.

The comparable results for the panel of older tasters are shown in Table 3, and it is noticeable that, unlike the younger panellists, the older group were not conscious that the fresh bovine cheese was physically different from the caprine/ovine cheeses. However, this conclusion does mask an obvious contrast with respect to chewiness: the value for the bovine cheese was 50% lower than the value for caprine/ovine (2), and the reason for this anomaly might be worth further study.

The mature tasters were more aware of the saltiness of the cheese, and detected correctly that the caprine/ovine (1) cheese had a lower salt content than the others. This same group was also aware that the caprine/ovine (2) cheese had a more liberal coating of mint (data not shown) than the other two

Table 3 Mean scores (%) for selected attributes of halloumi cheese given by nine panellists of average age 46

	Fresh halloumi cheeses			*Mature halloumi cheeses*			*Statistical analysis*	
Attributes	*Caprine/ Ovine 1*	*Caprine/ Ovine 2*	*Bovine*	*Caprine/ Ovine 1*	*Caprine/ Ovine 2*	*Bovine*	*Manufacturing procedure*	*Age of the cheese*
Texture								
Springy	55.4	63.7	53.9	24.2	16.1	21.8	NS	***
Moist	64.2	50.6	49.0	25.4	16.9	39.5	NS	***
Chewy	51.0	67.2	33.4	42.2	42.4	36.2	NS	NS
Crumbly	32.3	31.2	32.7	68.1	54.3	51.1	NS	**
Taste and flavour								
Salty	25.7	45.0	51.1	57.2	67.4	70.2	**	***
Bitter	7.5	13.4	22.1	38.5	46.7	26.7	NS	**
Acidity	9.6	15.9	26.5	59.8	63.8	51.2	NS	***
Creamy	47.0	37.9	44.1	46.0	20.9	29.2	NS	NS
Milky	58.8	44.1	44.1	39.3	37.3	41.5	NS	NS
Minty	17.8	40.9	11.4	15.9	18.0	10.5	***	*
Overall flavour and preference								
Hedonic	35.9	42.1	35.2	46.4	44.4	42.2	NS	NS
Flavour intensity	28.2	43.1	36.9	59.9	79.8	64.7	NS	*
Appearance								
Colour	39.7	51.5	36.9	57.0	64.4	21.8	***	NS
Body	55.9	50.7	31.5	35.3	53.4	32.6	NS	NS

$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

NS = no significant difference

brands. The absence of an overall preference for one cheese rather than another was not expected in light of the clear selection of the caprine/ovine (1) cheese by the younger panel, but the older group did detect a difference in colour between the cheeses.

This reaction to colour was pronounced on examination of the mature cheese, and the bovine cheese scored badly in comparison to the cheeses made from the goat/ewe's milk mixture. The general increase in flavour intensity was noted by the older panel, as were major increases in saltiness, acidity and, to a lesser extent, bitterness. Although no significant differences were noted in relation to the type of milk used, the panel noted the obvious changes in springiness and moistness with maturation and, in addition, felt that the mature cheeses were more crumbly than the fresh samples.

In considering the data in Tables 1 and 2, it is acknowledged that the lifestyles of the two groups will be different, but as none of the students had been in the UK for more than six months at the time of the sensory analysis, it seems feasible to suggest that they would not have lost their appreciation of halloumi cheese. It was assumed that no members of the groups were taking any medication, as even painkillers can alter an individual's perception of smell and taste.[14]

If these premises are true, then the data confirm that the older panel reacted to the three variants of halloumi in a manner different from the younger group of tasters. For example, the older panel was fully aware of the levels of mintiness in the fresh cheeses, whereas the younger group did not even become aware of the loss and/or masking of the mint flavour as the cheeses matured.

Perceptions of salt levels were again different for the two groups. In the fresh cheeses, for example, the responses of the younger panel was at variance with the compositional data, but the sensory values assigned by the older panel matched the expectations based upon chemical analysis. On the basis of age, this result might not have been anticipated, because Maruyama *et al.*[15] suggest that sensitivity to salt declines in men above 44 years old, and hence the younger panel should have been more responsive. One possible explanation for this result could be a threshold effect, in that some older people can only detect concentrations of NaCl double those noticed by teenagers.[16,17] Therefore, it could be that the ~3.0% concentration of NaCl in fresh halloumi 'swamped' the receptors of the younger panellists, impairing their ability to discriminate accurately between the levels, whilst the lower sensitivity of the older panel coped more readily. This point is borne out by the fact that, when examining the mature cheeses (tasted second), the responses of the younger group became more reliable, even though the cheeses had higher salt levels. Whether or not saturation of a proportion of the receptors diminished the overall impact of the salt for the younger group was not investigated, but 'adaptation' to the taste could be one feasible explanation.[18]

The clear preference of the younger panel for the fresh cheese was clear-cut, and it may be that a relative sensitivity to acidity was the crucial factor. Certainly, Zandstra and de Graaf[19] found that the younger panellists (mean age of 23 ± 2 years) were more sensitive to citric acid than the older panel (43 ± 5 years), and Jellinek[20] suggested that such reactions are a reflection of the higher concentrations of taste buds on the tongues of young people. However, Miller[21] has queried whether there is any significant decline in the number of taste buds with age, and suggests that the reasons for taste dysfunction are far more complex than those associated with simple anatomical considerations.

The build-up of flavour as a result of microbial activity in the brine and/or cheese was clearly noted by the older panel while, by contrast, the young panellists found all the cheeses highly flavoured. According to Schiffman and Warwick,[22] taste thresholds are 2.0–2.5 times higher in persons over 65 years of age compared with a young group and, although the average age of the older panel in this present trial was only 46, the same effect could have been operational. Whether a panel with an average age higher than 46 would have reacted differently to the halloumi cheeses could not be tested, but it may be relevant that the pattern described by Lawlor and Delahunty[5] suggests that exposure of a product to a panel with an average age of > 65 years, for example, would only exaggerate trend(s) revealed by a middle-aged group.

It was also notable that the younger panel rated some of the fresh cheeses very highly indeed—nearly eight out of 10 for the hedonic rating of the traditional ovine (1) cheese, which was more than double the rating given by the resident Cypriots. By contrast, the panel from Cyprus, who ate halloumi every day, were more critical (or at least conservative) in their overall ratings. Jellinek[20] proposed that older panel members give more precise ratings because they are inclined to concentrate on the task in hand better than 20-year-olds, and a similar trend was reported by Zandstra and de Graaf.[19] Nevertheless, the younger group remained slightly more conscious of a masking of the intensity of the creamy and milky flavour notes with maturation.

CONCLUSION

Clearly, the size of the panels—limited by the need for familiarity with the product—is too small for the conclusions to be other than tentative, but the results do support, to a degree, the contention of Cardello[23] that the age of a panel can affect the outcome of a trial. It was pleasing also that all the descriptors appeared to be appropriate for halloumi, and none of the panellists offered any additional or alternative terms.

What may be equally important, at least from a commercial standpoint, is the fact that although the preference of the young panel was for those products made from caprine/ovine milks, the cheeses made from bovine milk were rated as 'acceptable' by tasters of all ages. As typical consumers do not often have the opportunity to compare samples, it may be encouraging for the dairy industry in Cyprus that the change to using bovine milk is unlikely to generate any adverse reaction, even from residents of Cyprus who have eaten halloumi cheese for years.

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Microbiology of brines used to mature feta cheese

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The microfloras of brines from two commercial factories manufacturing traditional feta cheese were examined over a period of 60 days, which is the normal maturation time for Greek feta. Non-starter lactic acid bacteria were the dominant group, and Lactobacillus paracasei *ssp.* paracasei *and* Lactobacillus plantarum *were the principal species identified. A number of strains of* Debaryomyces hansenii *were isolated from the brines, and both the yeasts and the lactobacilli possessed enzymes capable of degrading fat or protein. It is suggested that these microfloras play an important role in the maturation of feta cheese. No recognized pathogens were isolated from the brines of either factory.*

INTRODUCTION

Feta cheese belongs to the family of white, brined cheeses that are characteristic of the Mediterranean and Middle East regions. It is a semihard, crumbly variety with a slightly aromatic flavour, and it is generally regarded as being Greek in origin;[1] indeed, in Greece, the word 'φετα' is often used as though synonymous with the word 'τυρι' (cheese). It is made in numerous factories across Greece with outputs ranging from a few hundred to several thousand tonnes of cheese per week. Traditionally, feta was made from raw ovine milk but, nowadays, mixtures of pasteurized ovine and caprine milks may be used and, in the larger factories, bovine milk alone may form the base material.

Standardization and pasteurization of the milk for feta production are normal practice, and starter cultures of *Lactococcus lactis* ssp. *cremoris* and *lactis* or *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are widely used to ripen the milk at 30–32°C.[2] The traditional sources of coagulant are the abomasa of lambs or kids,[1] but large factories prefer to use commercial rennets to coagulate the milk and lipase enzymes to provide a piquant flavour note to the final cheese. On coagulation, the gel is cut into 2–3 cm cubes and, after standing in the whey for 30–60 min (30–32°C) to become firm, the curd is ladled into moulds or 'bags' of cheesecloth to drain overnight (~20°C). The blocks of firm curd are then cut into pieces (1 kg) and dry salted. Finally, the salted blocks are stored for 2–3 weeks (prematuration stage) in large holding tanks (or small cans holding 10 kg of cheese) of brine (6–8% NaCl at 18–20°C). The final storage temperature of the cheese is ~5°C and, at this temperature, it will remain in the brine for a further 2–4 months prior to sale.

The microbiology of feta and related white-brined cheeses is well documented[2–4] and. typically during the prematuration stage for feta cheese, the population of non-starter lactic acid bacteria (NSLAB) increases significantly while the pH falls as a result of acid production.[4] Thereafter, microbial counts tend to stabilize for the remaining period of maturation (60 days at 5–7°C). If used, mesophilic starter cultures tend to die out early during maturation and, in teleme for example, lactococci were present only in the curd and in 5-day-old cheeses.[4] This decline in lactococci is probably due to the inhibitory action of the low pH and/or high salt-in-moisture values in the maturing cheese,[5] and the same factors are also reported to lead to a rapid reduction in the counts of staphylococci and coliforms.[6] The latter group of microorganisms is unaffected by the salt concentration in white-brined cheeses[7] and hence it is the acidity that is usually lethal.[8] Isolates of enterococci may also be present, but usually at low numbers in cheeses such as feta and teleme.[4] anthotyro[9] or nabulsi.[10] In feta and most brined cheeses, yeasts are not normally cited as being amongst the dominant microfloras.[11,12] but high levels of contamination have been observed.[3]

Although the microbiology of feta cheese is well established, few studies have been made of the brine employed for storage, and it is usually assumed that the microflora of the brine will be the same as that of the cheese. However, more recently the view has come under scrutiny on the grounds that pathogens, such as *Listeria monocytogenes* and *Escherichia coli* 0157:H7, have been associated with brined cheeses[13–15] and may survive for longer in the brine than in the actual cheese. If such survival is widespread, then there could be a strong case for sterilizing the

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brine before use, but any such treatment of commercial brines would have to acknowledge that many microbial changes occur in brine throughout the maturation period, and that at least some of these changes are likely to influence the flavour of the retail cheese.[16] Consequently, any means of removing pathogens from brine should leave this desirable microflora intact and, while such an approach might be feasible,[17] the first essential is to confirm which groups of microorganisms dominate the brine of a typical white-brined cheese.

Consequently, the initial aims of this project were: (i) to isolate the principal yeasts and lactic acid bacteria of non-starter origin in the brines of two different brands of feta cheese manufactured in Greece; and (ii) after identification, to characterize their enzyme profiles to determine whether or not they could play a role in the flavour and/or texture development of the cheeses in question.[18]

MATERIALS AND METHODS

Four tins of freshly manufactured feta cheese (made from ovine milk) were obtained from two different dairies (A and B) in Greece and air-freighted at ambient temperature to the laboratory in Reading, UK. For the prematuration stage, the cans were stored for 10 days at 18°C, but a cold store at 5°C was used for the remaining period of maturation. For the microbiological analyses, representative samples of brine were taken from each can on days 2, 10, 20, 30 and 60 of the maturation period by shaking the can gently and extracting the required samples (10 ml) with a sterile pipette. Four samples, two from dairy (A) and two from dairy (B), were taken for chemical analysis on days 15, 30 and 60.

Chemical composition

The gross chemical composition of the brine samples was determined using standard methods of analysis for total nitrogen, fat, salt, ash, lactose, total free fatty acids and pH.[19]

TABLE 1

Chemical composition of feta cheese brines at different stages during the maturation period[1]

	15 days		30 days		60 days	
Constituent	Dairy (A)	(B)	Dairy (A)	(B)	Dairy (A)	(B)
pH	4.4	4.1	4.5	4.1	4.5	4.2
Salt	5.5	7.8	5.6	7.9	5.6	7.8
Fat	Trace		Trace		Trace	
Protein	2.9	0.9	2.9	1.4	3.0	1.0
Ash	7.0	8.5	7.0	8.5	7.1	8.7
Lactose	2.0	0.5	1.1	0.3	0.1	0.3
VFFA	122	74	160	76	82	22

[1]All figures are means of duplicate readings and cited as g/100 ml of brine except for volatile free fatty acids (VFFA) which are given as mg/100 ml.

Microbiological analyses

At the time intervals mentioned above, samples of brine from each can were examined for: total colony count at 30°C,[20] coliforms,[21] *Enterococcus* spp.[22] and yeasts and moulds.[23] In addition, a total count for lactic acid bacteria was made on MRS agar, while *Lactobacillus* spp. were isolated separately on acidified MRS.[22]

Although their presence was not anticipated, there have been reports that certain pathogens can survive in brined cheeses,[7,13–15] so the samples of brine were also examined for coagulase-positive *Staphylococcus aureus*,[24] *Listeria* spp.[25] and *Yersinia enterocolitica*.[22]

Once the colonies on acidified MRS had been counted, 10 typical isolates were Gram stained and checked for the catalase reaction, production of gas from glucose and growth at 15°C. In addition, frequent isolates of *Lactobacillus*, as judged by colony morphology, were subcultured in MRS broth (30°C for 24 h) and characterized using the API 50 CHL strips.[26]

The yeasts were enumerated on yeast dextrose chloramphenicol agar[23] and a number of morphologically distinct isolates was subcultured and identified using the schemes suggested by Kreger van Rij,[27] Deak and Beuchat[28] and Tzanetakis *et al.*[29]

As both the species of *Lactobacillus* and the yeasts could be implicated in bringing about desirable flavour changes during maturation,[16] the cultures identified as dominant during the enumeration procedure were examined by the APIzym system to check for the production of proteolytic or lipolytic enzymes.[30–31]

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the brines is shown in Table 1. The salt and ash contents appeared to be stable in the brines from both dairies during the maturation period. There was, however, a marked contrast in the salt concentrations between the two brines. Although the pH of brine (A) was higher than that of brine (B), probably reflecting the buffering capacity of the higher protein content, the acidity may have increased slightly with time as the lactose in the brine was being slowly metabolized to lactic acid by the high counts of NSLAB in brine (A) (Table 2); in brine (B), the higher salt content and reduced lactose level could have tended to suppress microbial activity.

The crude protein content remained constant in both brines during the course of maturation, but it is interesting to note that the concentration of volatile free fatty acids slowly declined over the same period. This pattern suggests that biochemical changes of various types are occurring continuously in the brine throughout the maturation period, and it is likely that different substrates and/or proportions of substrates are being fermented at

TABLE 2

Changes in the dominant microfloras of two brines used to store feta cheese over a period of 10 days at 16°C and 50 days at 5°C[1]

Group		Time (days) 0	10	20	40	60
TCC	(A)	1.4×10^7	2.2×10^8	1.3×10^8	3.1×10^8	1.8×10^8
	(B)	1.4×10^8	8.0×10^7	3.0×10^7	5.5×10^7	2.4×10^7
Coliforms	(A)	96	<10	<10	<10	<10
	(B)	1.1×10^5	3.0×10^2	2.2×10^2	2.1×10^2	1.4×10^2
Enterococcus	(A)	<10	<10	<10	<10	<10
	(B)	9.8×10^2	6.8×10^2	2.2×10^2	1.2×10^2	6.7×10^2
LAB (total)	(A)	2.2×10^6	3.0×10^7	7.4×10^7	1.8×10^8	8.9×10^7
	(B)	1.5×10^6	2.6×10^7	1.2×10^7	2.5×10^7	1.8×10^6
Yeasts	(A)	2.4×10^5	1.1×10^5	1.4×10^5	1.3×10^6	1.8×10^6
	(B)	1.2×10^5	2.4×10^6	1.9×10^6	3.4×10^6	3.5×10^6

[1]All counts are mean number of colony forming units/ml of brine.
TCC, total colony count; LAB, lactic acid bacteria.

variable rates with. perhaps, different end-products being produced. These differences may result in the manufacture of retail products with slightly different characteristics.

Microfloras of the brines

Even though *L. monocytogenes*, *S. aureus* and *Y. enterocolitica* may survive in the harsh environment of feta cheese brines, no isolates were obtained from the brines tested. According to Gilmour and Harvey,[32] the main factors that influence the survival of *S. aureus* during maturation of cheese are the activity and quantity of the starter culture. nutrient uptake by the starter bacteria and the inhibitive capacity of certain NSLAB, and these same factors could also affect other pathogens.

Despite the unfavourable environment of the brine, high total counts were present in both brines. The brine of dairy (A) was found to have a mean total count of 1.4×10^7 colony forming units (cfu)/ml at the beginning of maturation, while the brine of dairy (B) had a mean total count of 1.8×10^8 cfu/ml; neither count changed significantly over the 60 days of maturation. These microorganisms may contaminate the brine from the surface of the cheese blocks, the environment, the water, the salt or from the inoculation of the fresh brine with mature brine (a common practice in the manufacture of traditional feta cheese). Since the brine is rich in nutrient substances migrating from the cheese, it forms a good medium for the specific microflora favoured by the prevailing ecological conditions.

Coliforms were found in the brine from both dairies on the first day of sampling. The brine from dairy (B) was found to have a high population of coliforms (estimate of 1.1×10^5 cfu/ml by the most probable number (MPN) method) but, while this number declined throughout the maturation period, it was notable that a residual count of 1.4×10^2 cfu/ml of brine remained even after 60 days. If this same count was present in the cheese, then the level would be unacceptable in most countries,[33,34] and it would have been of interest to establish which genera were so persistent; the more so, perhaps, as the pH of brine (B) was lower than that of brine (A). Low numbers of coliforms have been found in other cheese brines,[35] but usually when the brine was weak (< 6% NaCl) and the storage temperature was high (18–20°C). *Klebsiella aerogenes*, in particular, was found to be responsible for early blowing and poor cheese quality in white-brined cheese.[36] However, since coliforms are often used as an indication of postpasteurization contamination,[33,37] the low numbers in the brine from dairy (A) suggest that sound processing conditions exist in that factory.

Enterococci are also used as indicators of poor hygiene and/or possible faecal contamination in dairy products,[34,37,38] but *Enterococcus* spp. were detected only in the brines of dairy (B) at low numbers (9.8×10^2 cfu/ml) and the number remained constant throughout the maturation period. Macedo *et al*[39] related a high level of enterococci to poor hygiene during milk processing, but the resistance of enterococci to adverse conditions could also be relevant.[40] However, owing to their proteolytic activity and acid production, it has been suggested that *Enterococcus* species may play a beneficial role in the maturation of certain cheeses when they occur in high numbers. In addition, *Enterococcus faecium* and *Enterococcus faecalis* have been reported to produce bacteriocins that might alter any associated microflora.[41]

NSLAB formed the dominant microflora in the brines throughout the maturation period. The combined effect of low pH and high NaCl appeared to have a selective effect on the NSLAB and, as can be seen from Table 2, the numbers of NSLAB in the brines at 60 days were ~10^7 cfu/ml in dairy (A) and ~10^6 cfu/ml in dairy (B). Given the long maturation period of feta cheese, this group of microorganisms may make a critical contribution to the biochemical processes that take place prior to retail sale. In particular, NSLAB are able to transform lactic acid, citrate, proteins and fat into volatile compounds which, together with amino acids and other products from the degradation of caseins, play an important role in the development of cheese flavour.[42,43]

All the isolates from acidified MRS were Gram-positive, catalase-negative rods, unable to produce gas from glucose and capable of growing well at 15°C. An examination of some typical cultures was completed by monitoring their biochemical reactions on API 50 CHL strips.[26] The results are shown in Table 3, and these identified the most frequent isolate as *Lactobacillus paracasei* ssp. *paracasei*, with a probability of 99.6–98.7%. The remaining type of culture gave a low probability of identification with the API 50 CHL system as *Lactobacillus plantarum*. Some biochemical differences were noted between the strains of

TABLE 3

Some biochemical reactions of the isolates of *Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus plantarum* along with the reactions of the type culture[1]

	Lb. paracasei ssp. paracasei				Lb. plantarum	
	1	*2*	*3*	*Type*	*1*	*Type*
L-Arabinose	–	–	–	–	–	–
Ribose	+	+	+	+	+	+
D-Xylose	–	–	–	–	–	–
Galactose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Sorbitol	–	–	–	+	–	+
α-Methyl-D-mannoside	–	–	–	–	–	+
N-Acetyl-glucosamine	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+
Aesculin	+	+	+	+	+	+
Salicin	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Melibiose	–	–	–	–	–	+
Saccharose	+	+	–	+	+	–
Trehalose	+	+	+	+	–	+
Inulin	–	–	–	+	–	–
Melezitose	+	+	+	+	+	–
D-Raffinose	–	–	–	–	–	–
β-Gentibiose	+	+	+	+	+	+
D-Furanose	+	–	–	+	–	–
D-Tagarose	–	–	–	+	–	–

[1]All tests were completed using the API 50 CHL system.

Lb. paracasei ssp. *paracasei* tested, but these variations did not undermine the original identification. In addition, the presence of *Lb. paracasei* ssp. *paracasei* was not unexpected as it has been isolated from white-brined cheeses made from goats'[10,30] and ewes' milk,[4,10,39] as indeed has *Lb. plantarum*.

Nevertheless, differences between strains could be important in commercial practice for, if strains from within this microbial group are specific to a given dairy, they could impart a distinctive flavour profile to the finished cheeses. Thus, as shown in Fig. 1, the three isolates of *Lb. paracasei* ssp. *paracasei* examined by the API-Zym System for the activity of the certain enzymes each gave slightly different levels of reaction. The presence of aminopeptidases could be relevant with respect to the course of cheese maturation, but it was notable that none of the strains exhibited any esterase or lipase activity.[41]

While yeasts are not normally the dominant organisms in feta cheese,[4] they have been reported to constitute a major part of the secondary microflora in feta and other cheese brines as a result of environmental contamination;[45,46] few yeast cells survive pasteurization. The high salt concentration and low pH of most brines favour the growth of yeasts,[6] and a gradual increase in the number of yeasts was observed in the brines from both dairies, with numbers reaching ~10^6 cfu/ml in the brines at 60 days (Table 2). On the basis of colonial morphology (Table 4), selected yeasts were identified using the schemes suggested by Kreger van Rij,[27] Deak and Beuchat[28] and Tzanetakis *et al.*[29] Some of the results of these diagnostic tests are shown in Table 5 but, as with *Lb. paracasei* ssp. *paracasei*, strain differences within the species of *Debaryomyces hansenii* were evident (data not shown). The species of yeast isolated from the brines were identified as *D. hansenii*, *Candida versatilis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Yarrowia lipolytica*, but *D. hansenii* was found to be predominant. The presence of *Y. lipolytica* during the maturation stage could be significant because the proteolytic and lipolytic activities of this

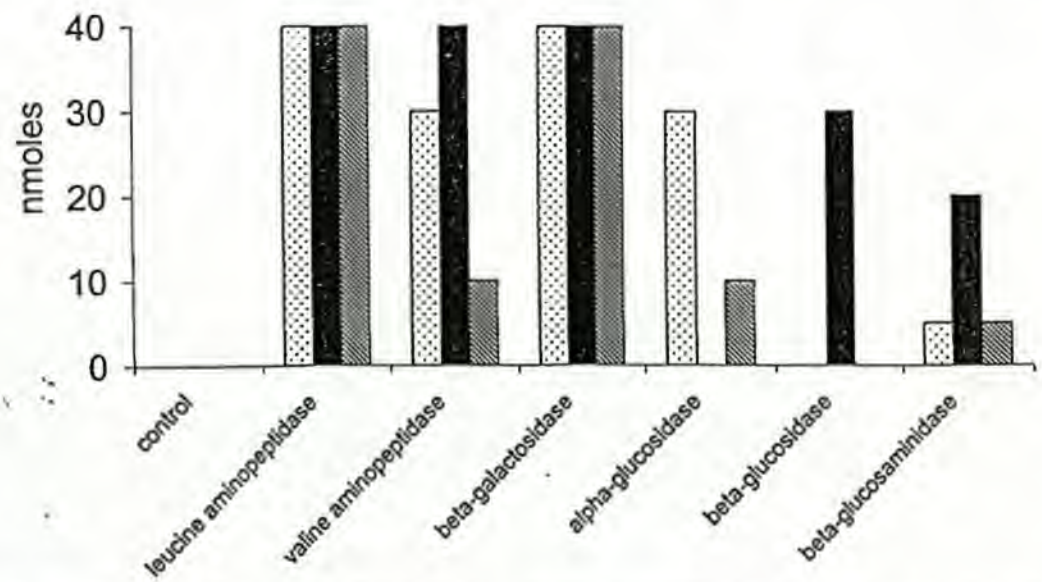

Fig. 1. Some biochemical reactions of three strains of *Lactobacillus paracasei* ssp. *paracasei* isolated from brines used for the storage of feta cheese over a 60-day maturation period.

TABLE 4
Morphological characteristics of yeasts isolated from feta cheese brines[1]

Species	Characteristics
Debaryomyces hansenii	Colonies: white, entire, smooth, dull, 1.5 mm diam. Cells: spherical
Candida versatilis	Colonies: white, entire, convex, dull, 1.5 mm diam. Cells: ovoid or spherical
Kluyveromyces marxianus	Colonies: cream, entire, 1.5–2 mm diam. Cells: ovoid
Saccharomyces cerevisiae	Colonies: cream, wrinkled, volcano-like, 1.5 mm diam. Cells: large, ovoid or spherical
Torulaspora delbrueckii	Colonies: white/transparent, convex, 2–2.5 mm diam. Cells: spherical and often in short chains
Yarrowia lipolytica	Colonies: white, dull, 1–1.5 mm diam. Cells: ovoid and some mycelial threads present

[1]All colonies were examined after growth on yeast dextrose agar at 25°C for 72 h.

species were much higher than those of the other isolates (Table 5).

Depending on the special characters of the yeasts, their population numbers and possible interactions with other microorganisms present in the brine, yeasts may have positive or negative effect on the quality of finished cheese. For example, the comparative levels of enzymatic activity revealed between the strains of *D. hansenii* could be important with respect to flavour (Fig. 2), as all the strains showed quite strong activity against the four-carbon substrate (β-napthyl-butyrate) and the eight-carbon substrate (β-napthyl-caprylate), indicating esterase and esterase/lipase activity, respectively. Strong leucine aminopeptidase activity was evident in all strains and, although valine aminopeptidase was weak or absent, the results suggest that the liberation of free amino acids might follow from growth of *D. hansenii*. β-Galactosidase was evident in all strains, and Tzanetakis *et al*[29] observed α-glucosidase activity in other strains of *D. hansenii* isolated from the surface of feta cheese.

The other species of yeast showed some differences with respect to enzyme activity (Fig. 3), but all species showed esterase, esterase/lipase and leucine aminopeptidase activity. Again, these yeasts, owing to their slightly proteolytic and lipolytic activity, could affect the flavour of the cheese directly,[47–49] or indirectly through the formation of substances which stimulate growth of the lactic acid bacteria. In addition, as some strains of *D. hansenii* fermented lactose, they could have an influence on the flavour/aroma of

TABLE 5
Some further diagnostic characteristics of the yeasts isolated from the brines used to store feta cheese

Species	N	Cel	M	Cyc	Cad	Ra	KG	37°	Pel	Trib	Gel	Milk	10	15%
Debaryomyces hansenii	–	–	+	+	+	+	nd	–	+	+	–	+	+	–
Candida versatilis	+	–	+	+	nd	nd	+	–	–	+	–	+	–	–
Kluyveromyces marxianus	–	–	–	+	–	+	nd	–	–	+	–	+	+	–
Saccharomyces cerevisiae	–	–	–	–	–	+	nd	+	–	–	–	+	–	–
Torulaspora delbrueckii	–	+	+	–	–	nd	nd	–	+	+	–	+	+	+
Yarrowia lipolytica	–	–	+	+	nd	nd	nd	–	+	+++	++	+++	+	–

N, nitrate assimilation; Cel, cellobiose assimilation; M, mannitol assimilation; Cyc, cycloheximide resistance; Cad, cadaverine assimilation; Ra, raffinose assimilation: KG, 2-ketogluconate assimilation; 37°, growth at 37°C; Pel, formation of pellicle in liquid media; Trib, lipolysis observed on Tributyrin agar; Gel, liquefaction of gelatin; Milk, proteolysis observed on milk agar (10%); 10%, growth in presence of 10% salt; 15%, growth in presence of 15% salt. All species were negative for erythritol assimilation and the urease reaction.
nd, not determined.

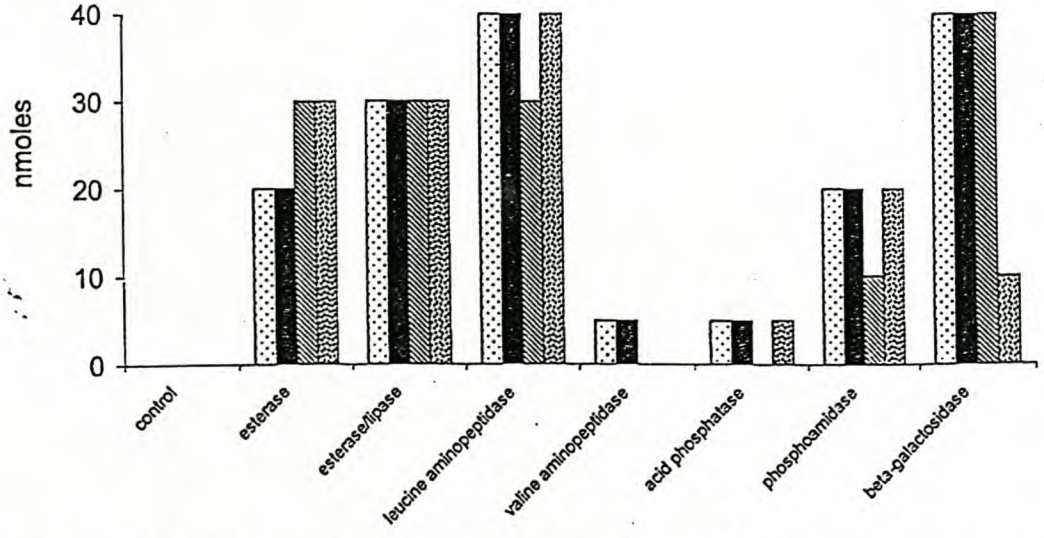

Fig. 2. Some biochemical reactions of four strains of *Debaryomyces hansenii* isolated from brines used for the storage of feta cheese over a 60-day maturation period.

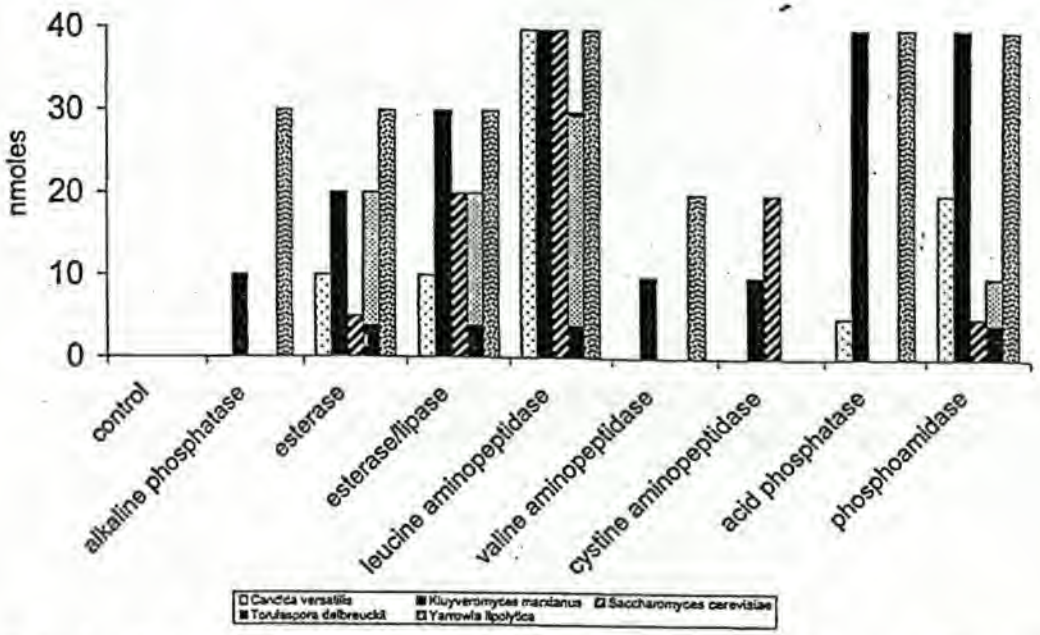

Fig. 3. Some biochemical reactions of four species of yeast isolated from brines used for the storage of feta cheese over a 60-day maturation period.

cheeses by producing volatile acids and carbonyl compounds.[13]

Antimicrobial activities of yeasts, including the secretion of 'killer factors' with broad antimicrobial spectra have been reported,[50] but whether or not such activity could be attributed to the yeasts in this study or could have influenced the survival of pathogens in the brines was not investigated.

However, preliminary trials (data not shown) suggest that the deliberate incorporation of selected yeasts into the storage brine for feta cheese has a beneficial impact on the organoleptic properties of the mature product. Careful species/strain selection has proved critical, for yeasts can also be responsible for defects associated with an unpleasant yeasty or ester-like odour.[48] In other circumstances, yeasts may be responsible for gas formation[50] and, in the case of white-brined cheeses, swelling of the cans can be caused by yeasts which ferment lactose to ethanol and carbon dioxide. Yeasts can also increase the pH of a medium and encourage the growth of *S. aureus* and possibly other pathogens and spoilage bacteria,[51] or give rise to a more general deterioration in quality as the pH of the cheese increases above 5.0.[32]

Conclusions

The overall properties of brine, including the presence of nutrients diffusing from the cheese, pH, biochemical changes taking place during maturation and possible microbial synergisms or antagonisms, give rise to a very special microbial ecosystem. These conditions selectively influence certain species to grow, survive or die according to their physiological and biochemical characteristics, and even in a cheese such as feta, which is manufactured from pasteurized milk using starter cultures, the microflora associated with in-brine maturation comprises a complex mixture of bacteria and yeasts.

Variations in the microfloras of the brines from different dairies can be attributed to the region of milk collection, differences in manufacturing procedures and the background microflora of the factory. Since such contamination is random and, perhaps, almost specific to a given dairy, the extent of biochemical changes such as proteolysis and lipolysis may vary from one dairy to another, so giving rise to subtle variations in the flavour of the retail cheese. The extent to which consumers are conscious of sensory differences between brands of feta has yet to be established, but it is evident that any attempt to employ sterile brine could dramatically alter the nature of the endproduct.

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A Research Note

Incidence of *Listeria* spp. in Retail Foods in the United Arab Emirates

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ABSTRACT

A survey of 1,101 samples of retail food items in the United Arab Emirates (UAE) covering dairy products, fresh vegetables, fresh/frozen meat and poultry and a range of "ready-to-eat" meals indicated that the incidence of *Listeria monocytogenes* was, in general, extremely low. Only in imported frozen chicken was *L. monocytogenes* detectable with a high degree of frequency, but fresh chicken and semi-processed meats of local origin were also contaminated. No indication of the number of organisms present in any given sample was sought, but as all the suspect foods would have been cooked prior to consumption, risks to the consumer should have been minimal. *Listeria* was not found in any "ready-to-eat" meals, including those made from chicken. Although *Listeria innocua* and *Listeria welshimeri* were detected in some retail foods, these species are normally regarded as being of little consequence with respect to public health.

Key Words: *Listeria*, methods, survey, retail foods.

Although four species of *Listeria*, namely *L. innocua, Listeria ivanovii, L. monocytogenes* and *Listeria seeligeri* have been implicated in diseases of man or other animals, the vast majority of human infections are caused by *L. monocytogenes* (*4*). In man, most people will be totally unaware that they may be harboring *Listeria* in their intestinal tracts, and any bacteria that enter the bloodstream are rapidly destroyed by the liver and, perhaps, the spleen (*3*). However, in susceptible groups, this normal resistance would appear to be deficient, and *L. monocytogenes* has been associated with outbreaks of listeriosis following consumption of coleslaw (*17*), pasteurized milk (*6*), Mexican-style fresh cheese (*9*) and a soft, surface-ripened cheese from Switzerland (*5*). Infection probably follows invasion of the intestinal tissues (*19*), but why some people escape infection is not clear, Similarly, it is not known whether onset of the disease is related to infective dose, but it is noticeable that the colony counts of *Listeria* in suspect foods can be extremely high; in the Swiss cheese mentioned above, *L. monocytogenes* was present at levels of around 1.0×10^7 colony-forming units (CFU)/g.

Equally important is the fact the *Listeria* are ubiquitous in nature, and *L. monocytogenes* in particular has, in addition to the foods cited above, been found in raw milk (*11*), ice cream, salad vegetables (*12,18*), several varieties of cheese as well as oven-ready poultry (*7,10*). This widespread occurrence of *L. monocytogenes* suggests that the poor storage of susceptible foods and/or cross contamination could lead to high colony counts being present at the time of consumption, and it was for this reason that the precautionary survey described in this paper was conducted.

Thus, while the UAE enjoy an excellent record with respect to food hygiene and no incidents of listeriosis have been documented, the volume of food imports from around the world is growing all the time, as is the range of locally-made products. Consequently, it was decided to examine a wide range of imported and local foods in order to establish:

(a) some indication of the incidence of *Listeria* spp. on retail items; and
(b) assess whether the level of contamination might pose any risk to consumers.

MATERIALS AND METHODS

Collection of samples.

During the actual survey, retail samples were taken of pasteurized milk (fresh and reconstituted), several varieties of local and imported cheeses, fresh vegetables: including sweet potatoes, bean sprouts, cabbages and tomatoes, raw meat, poultry and fish, frozen chickens and semi-processed meats, (e.g., meat balls and burgers), as well as a number of locally-prepared, "ready-to-eat" meals of Arabic or Western origin. The samples were collected at regular intervals over a period of 6 months and, after transfer to sterile bags, were returned to the laboratory in insulated containers; any foods not examined immediately were stored at <4°C for a maximum of 24 h.

Composite samples of the above foods were prepared by blending equal portions from three/five retail units either in a sterile container (liquid milk) or using a Colworth Stomacher. A test aliquot of 25 g was then removed and added aseptically to 225 ml of the appropriate enrichment broth (see below). In the case of

chickens, the composite sample was obtained by removing skin from the back, neck, breast, wings and thigh of the five retail units and macerating to an homogenous mass.

Isolation and identification of Listeria *spp.*

The most widely used approaches to the isolation and identification of *Listeria* spp. are those based upon the Food and Drug Administration (FDA) method for dairy products (*11*) and the U.S. Department of Agriculture (USDA) method for meat and meat products (*13*). The International Dairy Federation (IDF): (*8*) has also published a method for dairy products, and all these procedures include enrichment followed by isolation onto selective agar, confirmation of suspect colonies to generic level and, finally, identification of species by various biochemical tests.

In the present study, it was decided to employ the University of Vermont Medium (UVM I) - Fraser Broth method for all routine examinations involving meat, poultry, fish and "ready-to-eat" meals (Table 1), and *Listeria* Enrichment Broth (LEB) for examining all dairy products, as well as vegetables.

The composite samples (25 g) were added to the broths (225 ml) and, following enrichment (48 h at 35°C in LEB, or 24 h at 35°C in UVM I and Fraser Broth, respectively), loopfuls from either LEB or Fraser Broth were streaked onto the surface of Listeria Selective Medium (LSM - Oxford Formulation). The slightly elevated temperature of incubation for the enrichment broths (35°C as against 30°C recommended by the IDF [*8*]) was selected to comply with the advice relating to Fraser Broth (*1*), even though the lower temperature might have been more appropriate to resuscitate any cells that had been subject to stress (*15*).

The plates were examined after 48 h at 35°C for colonies producing black zones of aesculin hydrolysis. Colonies giving a "positive" reaction were then inoculated into Brain Heart Infusion broth for a motility test after 24 h at room temperature, and also streaked onto Tryptone Soya Yeast Extract Agar (TSYEA) to provide colonies for gram staining and the catalase and oxidase tests. Colonies from LSM that consisted of catalase-positive, oxidase-negative, gram-positive, short bacilli with tumbling motility were further examined for Beta-haemolysis and the CAMP reactions. Final identification as to species was carried out employing the Analytab Products (API) *Listeria* System (*2*).

RESULTS AND DISCUSSION

A total of 431 samples of dairy products were examined (Table 1), and all the samples of pasteurized milk were negative for *Listeria* spp. In contrast, four samples of imported white-brined cheese were found to be positive, and two of the isolates were confirmed as *L. monocytogenes*; the remaining two isolates were found to be *L. innocua*. The ability of *Listeria* to survive in brined cheeses has been reported elsewhere (*16*), and hence a low level of incidence (2.0% in the present survey) is not unexpected. It is of note also that, while two of the positive samples were available as "loose" cheese, the others were taken directly from cans sealed by the manufacturers; whether this latter observation indicates that raw milk was used in production or post-pasteurization contamination is not clear. However, the overall incidence was favorable in comparison with reports from elsewhere (*14*), where rates of contamination for Western-style cheeses were up to 7%. Hence while cheese remains a possible vehicle for *Listeria*, the risk to consumers in the UAE would appear to be below average. The situation with fresh vegetables was encouraging in that no isolates of *L. monocytogenes* were found in any of the 183 samples tested, and only four positives for *L. innocua* were recorded. This apparent absence of *L. monocytogenes* confirms the view that fresh vegetables are unlikely to be a source of listeriosis (*12*). Obviously, the use of contaminated manure on fields can change the position dramatically (*17*), as can the chopping and mixing associated with the production of pre-packed salads. However, the actual level of contamination in pre-packed vegetables should still be too low to cause real concern, and it has been suggested that <100 CFU/g of *L. monocytogenes* in mixed salad vegetables would be an acceptable standard (*12*).

Out of 70 samples of raw meat examined (Table 1), seven were positive for *L. innocua*, and the same organism was isolated twice from samples of fresh fish. Fresh chicken was found to be more prone to contamination, and ten out of 30 samples tested positive; *L. monocytogenes* and *L. welshimeri* were each isolated from one sample, and the remaining eight "positives" were *L. innocua*.

TABLE 1. *Results of a survey for the presence of* Listeria *spp. in different foods on sale in the United Arab Emirates (Feb. 93-July 93).*

Category	No. of samples analyzed		No. of positive samples	
	Imported	Local	Imported	Local
Dairy Products				
Fresh and reconstituted milk (pasteurized)	-	182	0	0
Cheese	196	53	4	0
Total	(431)		(4)	
Fresh Produce				
Vegetables	84	99	2	2
Total	(183)		(4)	
Meat, Poultry, Fish				
Raw meat				
beef	15	-	1	-
goat	17	-	1	-
sheep	24	-	5	-
camel	-	14	-	0
Fish	-	44	-	2
Fresh chicken (5 U/sample)	-	30	-	10
Total	(144)		(19)	
Frozen Products				
Frozen chicken (5 U /sample)	39	-	32	-
Semi-processed meat	-	107	-	34
Total	(146)		(66)	
Ready Meals				
Traditional foods	-	90	0	0
From restaurants	-	107	0	0
Total	(197)		(0)	

The examination of imported frozen chicken found that 32 out of 39 samples were contaminated with *Listeria*, with 18 of the 32 "positives" being confirmed as *L. monocytogenes*. This latter organism was also isolated from 12 out of 107 samples of frozen semi-processed meat products, while *L. welshimeri* was found in two samples and *L. innocua* in a further 20. Whether or not the high level of contamination of frozen chicken (82%) as against 33% for fresh product is a reflection of the contrasted sources of supply or differences in processing was not established, but clearly poultry can act as a significant carrier of *L. monocytogenes*. However, the fact that these retail items will be cooked before eating should eliminate any direct risk to consumers, for only extensive cross-contamination of other foods could lead to cell counts capable of causing disease.

The absence of contamination in any of the 197 "ready-to-eat" meals suggests that, as long as sound methods of food handling are employed in the home, "prepared foods" need not pose any risk for the consumer – at least as far as *Listeria* spp. are concerned.

A point emphasized, perhaps, by the fact that the meals included dishes based on chicken and semi-processed meats, as well as prepared salads. In other words, the range included foods where the raw materials might, on the basis of the survey, have been anticipated to be reservoirs of *Listeria*, as well as foods which, under conditions of poor hygiene, could have been subject to cross-contamination, and yet the microbiological quality of the sampled products was excellent.

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Review

Existing and potential applications of ultraviolet light in the food industry – a critical review

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Abstract: Short-wave ultraviolet light (UVC, 254nm) can reduce dramatically the microbial load in air or on hard surfaces free from food residues, and can eliminate pathogens from potable water filtered to remove organic residues and 'clumps' of bacteria. More recently, approval of the Food and Drug Administration (USA) has been sought for a system for the destruction of pathogenic bacteria in fruit juices using UVC, and the same approach could perhaps be applied to remove spoilage organisms from cider or wines. In contrast, long-wave UV light (UVA, >320nm) has limited microbiocidal properties, and for practical applications its effectiveness has to be enhanced by the presence of photosensitive compounds (eg furocoumarins) that will diffuse into a microbial cell prior to irradiation. The penetration of UVA into water is better than that of UVC, and its bacteriocidal action in the presence of photosensitisers can be rapid. However, pure furocoumarins are expensive and their addition to foodstuffs might be questioned on safety grounds.

Keywords: ultraviolet light; microbiocidal action; furocoumarins; water and fruit juice treatments

INTRODUCTION

Food safety is one of the most important issues facing the food manufacturing and service industries, for as consumers demand an increasing variety of ready-to-eat meals or dishes on a menu, so the risk of microbial contamination of an ingredient or the finished meal increases. The application of HACCP (hazard analysis critical control point) systems, heat treatments and efficient cold chains helps to reduce the opportunities for pathogenic micro-organisms to gain access to a food and/or grow to levels that will pose a risk from infection or toxin production, but, even so, the number of incidents of food-borne disease continues to rise in most industrialised countries.

Reversing this trend will not be easy, and yet many restaurants serve hundreds of meals per day without incident, and many food factories have equally commendable records with respect to hygiene. Obviously there may be many reasons why a particular company exposes the consumer, on occasions, to microbiologically unsafe products, but any procedure that could help to improve the situation must be welcome. One such procedure could involve the irradiation of food contact surfaces, rinsing water for food or process plant or air over a food preparation area with short-wave ultraviolet (UV) light, for the equipment is relatively inexpensive, the technique is, subject to certain safety precautions, easy to use and the radiation is lethal to most types of micro-organism. Whether the technique could or should be more widely applied in food preparation or production areas is a matter for speculation, as are the possible beneficial roles of long-wave UV light. Consequently, the aim of this present review is to consider some of the current applications of UV radiation in the food industry, and attempt to assess whether the microbiocidal effects of UV should be exploited further.

NATURE OF UV RADIATION

Ultraviolet (UV) light occupies a wide band of wavelengths in the non-ionising region of the electromagnetic spectrum between X-rays (200nm) and visible light (400nm). For practical purposes the UV spectrum can be subdivided into three regions:

- short-wave UV (UVC) with wavelengths from 200 to 280nm;
- medium-wave UV (UVB) with wavelengths from 280 to 320nm;
- long-wave UV (UVA) with wavelengths from 320 to 400nm.

The intensity of UV radiation is expressed as irradiance or intensity flux ($W m^{-2}$), while the dose,

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which is a function of the intensity and time of exposure, is expressed as radiant exposure ($J m^{-2}$).[1]

SOURCES OF UV RADIATION

Solar radiation

The sun emits radiation across a wide range of wavelengths, but the relative intensities of ultraviolet radiation reaching the earth's surface depend, to a considerable extent, on attenuation by the atmosphere through absorption and scattering. UVC is completely absorbed in the upper and middle atmospheres by ozone and molecular oxygen, but, while UVB is similarly attenuated, some UVB does reach the surface—much to the delight of sunbathers! However, UVA is barely affected, and hence the terrestrial environment is exposed mainly to ultraviolet radiation between 290 and 400 nm.[2] The intensity flux of UVA is about 35–50 $W m^{-2}$ at sea level,[3] and under these conditions a dose of 200 $kJ m^{-2}$ will be delivered over about 1 h of exposure. As a consequence, potentially lethal photoproducts can be formed at a considerable rate, and life in the open air would not be possible without the action of repair processes that ensure a drastic reduction in the damage caused by UVA.[4]

Artificial sources

Long-wave UV lamps

The light from mercury vapour lamps can be filtered to remove the visible spectrum and give an emission that is primarily UVA.[5]

Medium-wave UV lamps

Mercury vapour lamps are sometimes designed with pressures that produce maximum radiation in the UVB region, and using glass bulbs that freely transmit this energy.

Short-wave UV lamps

Mercury lamps designed to produce energy in the germicidal region (254 nm) are electrically identical to fluorescent lamps, but they lack the phosphor coating, and the use of glass allows the transmission of UVC. It should be noted that radiation below 260 nm will produce ozone which has to be monitored to prevent a hazard to health; a working atmosphere should not contain more than 0.2 $mg l^{-1}$ of air.

SHORT-WAVE UV RADIATION (UVC)

Impact on living cells

UV radiation in the range of 250–260 nm is lethal to most micro-organisms, including bacteria, viruses, protozoa, mycelial fungi, yeasts and algae. The relationship between germicidal effect and wavelength is illustrated in Fig 1, which shows the maximum effect at 254 nm and a fall to practically zero at 320 nm; in fact, the effectiveness at 320 nm is 0.4% of the peak value.

The damage inflicted by UVC probably involves

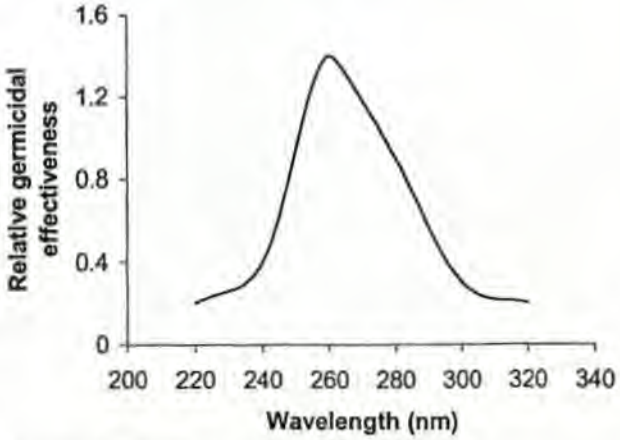


Figure 1. Direct lethality of UV wavelengths (after Ref 6).

specific target molecules,[7] and a dose in the range from 0.5 to 20 $J m^{-2}$ leads to lethality by directly altering microbial DNA through dimer formation. The main types of photoproduct in UV-irradiated DNA are cyclobutyl-type dimers (pyrimidine dimers), pyrimidine adducts and DNA–protein cross-links.[4] Purines are approximately 10-fold more resistant to photochemical alteration than are the pyrimidines, and because of this difference in sensitivity it has been implied that the photochemistry of the purines is not important biologically; by the time a significant amount of purine damage has occurred, the cells would have been inactivated by pyrimidine damage anyway.[8]

Once the DNA has been damaged, the micro-organisms can no longer reproduce and the risk of disease arising from them is eliminated. Temperatures between 5 and 37 °C have little, if any, influence on the microbiocidal action of radiation,[9] but moisture exerts a very marked effect. Where bacteria are suspended in air, an increase in relative humidity results in a greatly reduced death rate, especially at humidities greater than about 50%. Similarly, bacteria suspended in a liquid medium are much more resistant than those suspended in air, even after making allowance for the absorption of the medium.

Practical applications

The applications of the germicidal effects of UV fall into three broad categories: (a) inhibition of micro-organisms on surfaces; (b) destruction of micro-organisms in air; (c) sterilisation of liquids.

Disinfection of surfaces

The first category under this heading includes the sterilisisation of packaging materials, eg containers, wrappers or bottle caps, by arranging appropriate lamps over conveyors. The success of this application depends on the material surfaces being clean and free from any dirt which would absorb the radiation and hence protect the bacteria.

During the manufacture of aseptically filled UHT

dairy products, for example, UV sterilisation has been applied to the foil caps of HDPE bottles[10] and to cartons for liquid products.[11] Similarly, the Hamba BK10010/10 has been used for aseptic yoghurt filling, and all the packaging materials, eg plastic cups and aluminium foil lids, are sterilised using UVC lamps working at 100–200 mW cm^{-2};[12] the shelf-life of fruit yoghurt packaged in containers sterilised by UVC lamps was extended by about 2 weeks at 5–7 °C. The disinfection of working surfaces in food preparation areas could merit serious assessment as well, but the limiting factor could be the presence of irregularities which would protect bacteria from incident UV.

Short-wave UV can also be employed to treat the surface of an actual food. For example, it has been used to control food spoilage micro-organisms such as *Bacillus stearothermophilus* in thin layers of sugar[13] or *Pseudomonas* spp on the surface of meat.[14] However, meat that has been exposed directly to UV light sometimes develops off-flavours, and a similar problem has been encountered with milk. It has been suggested that these undesirable flavours arise owing to absorption of ozone and oxides of nitrogen, as well as to direct photochemical effects on the lipid fractions of milk or meat. These latter effects can be reduced by filtering-out the shorter wavelengths or covering the product with a layer of inert gas prior to irradiation,[9] but in any event there appears to be no evidence that any of the photoproducts are harmful to humans.

Fresh fish is another product with a superficial flora of *Pseudomonas* spp, and Huang and Toledo[15] demonstrated the effectiveness of reducing initial bacterial counts, using UVC irradiation, in prolonging the storage life of fish. Kuo *et al*[16] showed that UVC radiation is effective in reducing the total aerobic and mould counts, along with *Salmonella typhimurium*, on the surfaces of egg shells; this latter treatment may be of little practical use, as the more important pathogen with respect to hen's eggs, ie *Salmonella enteritidis*, would be inside the egg and protected by the shell. In the baking industry, contamination of fresh products with mould spores has always been a problem, but, with bread, irradiation of the loaves as they emerge from the oven is reported to extend significantly their shelf-lives.[6]

A combination of UVC radiation and heat has been suggested by Tanaka and Kawaguchi[17] for the production of high-quality raw meat. More specifically, the same authors envisaged that: (a) retail portions of meat could be vacuum-packed using a membrane that transmits UVC; (b) the surface of the meat would be sterilised with UVC; (c) the membrane would then be heat-shrunk using water at temperature sufficient to kill any bacteria that had survived the UV treatment; and (d) the meat would then be cooled rapidly to maintain quality.

Given the growing demand for 'organic' foods, the potential use of UVC as an alternative to fungicides for the control of post-harvest diseases of stored vegetables such as carrots has attracted attention.[18] For example, a pre-storage treatment of carrots with UVC induces the accumulation of the phytoalexin 6-methoxymellein (an isocoumarin), and this change increases tissue resistance to fungal pathogens.

Disinfection of air

In hospitals, UVC lamps have been used to create a curtain or barrier of radiation through which air must pass before reaching patients sensitive to infection, and UV radiation at 254 nm and 0.25 W m^{-2} has been used in the United States since the 1930s to decrease the number of air-borne bacteria in operating theatres.

For the handling of sensitive foodstuffs, a system which combines a laminar flow of air through filters to remove particles of size > 0.1 μm, and the use of UV radiation to kill any live micro-organisms that remain, has been suggested for the provision of clean sterilised air in the workplace.[19] Similarly, the microbiological quality of mechanically peeled fruit and vegetables is improved when UV-treated air is blown through the peeling unit counter-current to the flow of product.[20] The microbiological quality of air in cold stores can also be improved using an air sterilisation unit,[21] and the same technique has been applied to the air in egg hatching cabinets.[22]

Disinfection of liquids

Treatment with UVC is one of the simplest and most environmentally friendly ways of destroying a wide range of micro-organisms in water.[23,24] It has been used to disinfect sewage effluent, drinking water and water for swimming pools, and the combination of UV and ozone has a very powerful oxidising action which can reduce the organic content of water to extremely low levels.[2]

As UVC disinfects without any change in colour, flavour, odour or pH, it is an effective means of ensuring that drinking water is microbiologically safe;[25] the normal performance criterion is based on a 99.999% reduction of micro-organisms with a treatment time of < 1 min. The major limitations on the effectiveness of UVC radiation in this context are the following.

- Lack of penetration: in distilled water, UV radiation at 254 nm will have suffered a 30% loss in intensity 40 cm below the surface, while sea water will cause the same reduction over ~10 cm; a solution of sucrose (10%) or a natural spring water containing high levels of iron will cause the same loss within 5 cm.[25]
- In natural water supplies, any suspended solids must be filtered-out prior to treatment, and occasional 'clumps' of bacteria can pose a similar problem, ie the outer cells protect the more deep-seated ones. However, as small-scale water filtration units have become more efficient, so the ability of UVC systems to generate safe, potable water supplies has improved. In Japan, UVC radiation has been used for disinfection of natural mineral water with

no effect on the mineral content of the water or generation of taints or off-flavours;[26] the eradication of *Enterococcus faecalis* was the essential aim of the latter process.

In some food processing industries the situation may be rather different, and a simple reduction in the microbial load in a water supply may be more than adequate. For example, in the brewing industry a treatment that does not alter the taste or quality of the end-product is essential, and a number of breweries have become major users of UV disinfection systems.[27–29] Dosages in brewery water treatment can be quite high to ensure the absence of any spoilage problems during the early stages of the brewing process, and doses range from 300 to 600 Jm^{-2} compared with 200 to 300 Jm^{-2} for the treatment of potable water.

Similar applications tend to be limited by the lack of penetration of UVC into liquids containing organic matter, but the disinfection of the brine used to salt Mozzarella cheese has been proposed,[30] thus enabling spent brine to be reused rather than replaced. The brine has to be filtered to remove any cheese residues before treatment, and, after replenishing the level of NaCl, it is pumped back to the storage vats containing the cheese.

The treatment of more opaque liquids is clearly a problem, and yet Lodi *et al*[31] used UVC to reduce the total colony counts in samples of caprine milk by 50–60%, along with a specific fall in coliform counts of 80–90%. However, while these latter reductions could be valuable to prevent on-farm spoilage of milks with high bacterial counts, the presence of 10% of the original coliform populations would still render the milk unacceptable for human consumption. Whether or not the process could be made sufficiently reliable to replace pasteurisation for milk is an open question, but it may be relevant that, at one time, milk in Germany and North America was treated with UVC to enhance the concentration of vitamin D.[32] The most successful system was the Lembke plant in which turbulent flow was achieved by pumping milk at high velocity through transparent tubes of 1 cm diameter, and, as 80% of the UV radiation reached the milk, it was found that over 99% of bacteria initially present in the milk could be destroyed.[32] It is worth noting though that the keeping quality of such milk was worse than that of heat-pasteurised milk, even though the latter had a higher microbial count, and this anomaly was ascribed to the selective survival of coliforms.

More recently, it has been reported that the Food and Drug Administration (USA) is considering allowing UVC to be used to eliminate pathogens from fruit juices.[33] The alleged success of the system depends on ensuring that the flow of the juice is turbulent rather than laminar, holding the temperature of the juice below 5 °C and applying a rigorous HACCP programme. It is suggested also that this 'light-processed' juice retains its levels of vitamins A, B, C and E, and other processors of liquid products may well monitor this development with interest. However, as unpasteurised fruit juice has been recorded as a source of infection from *Escherichia coli* O157,[33] the comments of Burton[32] about the ability of coliforms to survive UCV treatments could be pertinent, as could earlier reports on the treatment of cider[34] and maple syrup.[35] In both cases the authors recorded reductions in microbial counts following UVC treatments, but no attempt appears to have been made to identify which genera survived.

One final barrier to the use of UVC for destroying pathogens in liquids appears to be the absence of any test to confirm that a specified treatment has taken place. Thus pasteurised milk can be subjected to the classic alkaline phosphatase test,[36] other heat treatments below 100 °C can be monitored by the acid phosphatase test,[37] but how can a Public Health Authority check a UVC-treated product? The author of Ref 33 suggests that records of product flow rates and UV emissions should be sufficient to ensure product integrity, but then a chart from a temperature recorder does not confirm that raw milk is not contaminating pasteurised product through a damaged gasket.

LONG-WAVE UV RADIATION (UVA)

Impact on living cells

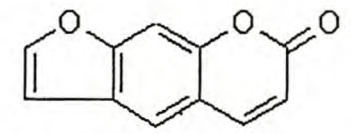
As UVA is poorly absorbed by living cells compared with UVC, little attention has been paid to any potential biocidal role. However, remedies of sunlight and herbs have been used for thousands of years to treat dermatological conditions such as psoriasis, a practice that confirms that some penetration of the skin cells does occur. Similarly, UVA does affect microbial cells, but it is far less effective than UVC. For example, the incident energy required to bring about a 50% reduction in microbial counts was 5 Jm^{-2} using UVA, whereas UVC achieved the same impact with only 10^{-5} Jm^{-2}.[38] Nevertheless, given that UVA is much safer for an operator to use than UVC, eg less risk of damage to the eyes if protective goggles are defective, interest in the sterilising effect of UVA has recently been revived.

The mode of action of UVA within cells is significantly different from that of UVC,[39] and the most likely effect(s) of UVA on micro-organisms are through:

(a) membrane damage—unsaturated fatty acids are readily oxidised to hydroperoxides, thus inducing changes in membrane permeability;[40]
(b) an oxygen-dependent reaction involving endogenous photosensitizing pigments—this mechanism involves the absorption of light by chromophores, resulting in their excitation, followed by reaction with oxygen to form active oxygen species or H_2O_2 which may be the primary agents of cell damage;[3,7,40] the latter compound has been impli-

cated as H_2O_2 pretreatment of cells of *E coli* induced resistance to UVA, probably because a repair system specific to oxidative damage was induced.[41]

A large number of compounds commonly present in micro-organisms have been suggested as possible endogenous target molecules, but the low lethality of UVA against micro-organisms means that it has little practical value unless the rate of kill can be enhanced by means of exogenous photosensitisers absorbed into the cell.[42] One group of compounds that meet this requirement are the tricyclic furocoumarins (see Fig 2), which are formed by the fusion of a furan ring with a coumarin molecule.[44] In general, Gram-negative bacteria are more resistant to hydrophobic antimicrobial substances (eg furocoumarins) than are Gram-positive species, principally because the outer cell membrane of the former contains lipopolysaccharides which can delay, or perhaps prevent, the entry of hydrophobic molecules into the cell.[45,46] Consequently, it may be that the hydrophobic furocoumarins are largely retained in the outer cell membrane of Gram-negative bacteria, and cannot diffuse into the cell to react with the DNA.[47] In addition, since the effectiveness of furocoumarins as antimicrobial agents depends on contact with the DNA, their distribution within a cell and interactions with other components (ie proteins) may also influence their antimicrobial activity.[48]



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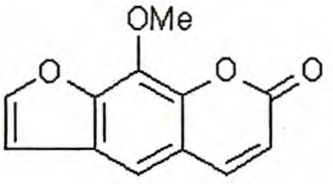

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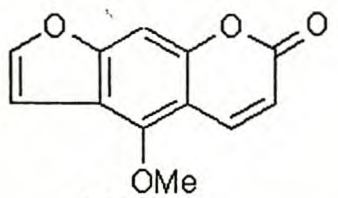

(III)

Figure 2. Chemical structure of some linear furocoumarins: (I) psoralen; (II) bergapten; (III) xanthotoxin (after Ref 43).

Potential use of the UVA/furocoumarin system

Antimicrobial activity

The furocoumarins are best known for their use in medicine, and a combination treatment involving 8-methoxypsoralen and sunlight/UVA radiation has found success in the control of psoriasis.[49] In a different context, Lin *et al*[50] employed 5 μg ml^{-1} of 8-methoxypsoralen with UVA to kill bacteria in human platelet concentrates required for transfusions.

Unlike the situation where UVA stimulates endogenous target molecules, the activated furocoumarins form cross-links between complimentary strands of DNA, so preventing the strands from replicating.[43] In addition, UVA plus furcoumarin produces DNA monadducts which damage both eucaryotic and bacterial cells, but the relative lethal impacts of cross-link formation or monoadduct action may vary.[51] The amount of furocoumarin needed to stimulate this reaction is very small, and, in a model food system under UVA illumination, Ulate-Rodriguez *et al*[52] tested the antimicrobial properties of linear furocoumarins at levels of 2–53 μg ml^{-1} against *Listeria monocytogenes, E coli* O157:H7 and *Micrococcus luteus*. *L monocytogenes* was inhibited, but *E coli* O157:H7 and *M luteus* were found to be more resistant; considerable variation in sensitivity has been found even with a single species.[6] More recently, Bintsis (unpublished) found that *L innocua, E coli* and *Staphylococcus aureus* suspended in tubes of Maximum Recovery Diluent (MRD Code No CM733, Unipath Ltd, Basingstoke, Hants, UK) (5.0×10^6 colony-forming units (cfu) in 10 ml) were inactivated rapidly by UVA and psoralen (see Table 1), whereas *Yarrowia lipolytica* and *Debaryomyces hansenii* (5.0×10^5 cfu in 10 ml) were slightly more resistant.

These figures confirm that the UVA/furocoumarin system can have a dramatic microbiocidal impact. However, it was recorded in a separate trial (no micro-organisms present) that the loss of irradiance through

Table 1. Inactivation of different types of micro-organism following a 60 s treatment with UVA[a]/psoralen (5 μg ml^{-1})

Micro-organism	*% killing*
Listeria innocua	99.8
Escherichia coli O157:H7	99
Staphylococcus aureus	99.9
Debaryomyces hansenii	97.5
Yarrowia lipolytica	82.7

[a] The experiment was performed with a Black-Ray Display Lamp (XX-15BLB) (Ultraviolet Products, Cambridge, UK) with the surface of the liquid at a distance of 10 cm from the lamp. At 10 cm the intensity was 45 W m^{-2}.

MRD was 34% over 5 cm, while in a simulated cheese brine including casamino acids (1.0%), lactose (0.5%) and NaCl (6.0%) the loss was approximately 95% over 5 cm. As neither tap water nor sucrose solutions (<10%) caused any loss of irradiance under the same conditions, it may be the peptides and amino acids in the MRD and brine that absorbed the radiation. For practical applications this point needs further study, because it has been reported elsewhere that UVA has the advantage over UVC in that amino acids do not absorb UV at wavelengths > 300 nm.[4]

Potential applications in food processing

Natural furocoumarins have been isolated from five plant families, namely the *Umbelliferae* (eg celery, parsley and parsnip), *Rutaceae* (eg bergamot fruit and lime), *Moraceae* (eg fig), *Leguminosae* and *Orchidaceae*. Typical concentrations of furocoumarins are shown in Table 2, and, while psoralen is the most photoreactive, it is likely that initial addition rates could be calculated on the basis of total furocoumarins; the wide variation in concentrations within the same species is a reflection of differences between cultivars, season/location of collection and method(s) of analysis. However, it should be borne in mind that if the UVA/furocoumarin system was to be used rather than UVC to sanitise a cheese brine, for example, each litre of cheese brine would have to be dosed with a minimum of 5000 μg of furocoumarin prior to irradiation, so that some of the concentrations in parsley or celery are clearly too low to be of practical or economic value. Furthermore, a number of studies have highlighted the fact that handlers of celery are prone to light-induced dermatitis due to UVA/furocoumarin interactions.[63–65] Consequently, although it might be attractive from a marketing standpoint to employ natural plant materials as a source of furocoumarins, the practical hurdles may prove insurmountable.

Nevertheless, it remains feasible to suggest that a combination of UVA and photosensitisers could be used to increase the shelf-life of perishable products, with the furocoumarins being incorporated, perhaps, into the packaging materials. If these same compounds then diffused into microbial contaminants on the surface of a product, they could be sensitised by natural light.[66] The direct addition of furocoumarins to foods could be a further option, but on the negative side it is important to highlight the facts that: (a) pure furocoumarins such as psoralen are expensive; and (b) although in the treatment of psoriasis the daily dose of 8-methoxypsoralen is about 20 mg, there is a recognised toxicological risk to the patient.[67] In particular, the ingestion of natural furocoumarins has been linked with the onset and/or development of cutaneous carcinomas,[68–70] and hence this risk alone will prevent their commercial exploitation within the food context.

If the phototoxic side-effects could be eliminated, then the UVA/furocoumarin system might be worth further evaluation, and it could be relevant that a number of synthetic furocoumarins are available that have the same therapeutic activity as 8-methoxypsoralen but, at least in mice, induce no toxic or carcinogenic reactions.[68,71,72] At present, the cost of such compounds would prohibit their use as components of any food preservation system.

Practical applications of UVA alone

Detection of chemical residues

The principal application of UVA within the food industry has been in relation to quality control, particularly for the detection of aflatoxins from *Aspergillus flavus* or *Aspergillus parasiticus* on various grains and nuts, eg maize, cottonseeds or peanuts, during storage. The aflatoxins have absorption maxima around 360 nm, and while aflatoxin B fluoresces blue at 425 nm, aflatoxin G produces a green–blue emission at 450 nm; these reactions can be employed to detect low levels of aflatoxin.[73] UVA is also reported to degrade aflatoxin M_1 in milk.[74,75]

In some stores and shops it may be necessary to check for the presence of rodents, and while dry rodent urine (fresh) glows blue–white under UVA, older deposits give a yellow–white glow. Rodent hairs also glow blue–white and are easily identified on sacks or intermixed with food grains.[76]

In the dairy industry, fresh deposits of milk-stone—a long-enduring problem—will fluoresce a strong yellow–white/bright blue–white under UVA.[77]

Detection of micro-organisms

The rapid identification of coliform bacteria in water is essential to ensure that public drinking water is safe,

Table 2. Some reported furocoumarin contents in various plants of the *Umbelliferae* and *Rutaceae*; all figures as $\mu g\,g^{-1}$ on a fresh or dry weight basis (see footnotes)

Plant	Psoralen[c]	Xanthotoxin[c]	Bergapten[c]	Total linear furocoumarins	Reference
Celery[a]	0.01–4.18	0.08–16.86	0.46–28.51	0.56–49.84	3–57
Lime peel[b]	14 ± 2	42 ± 6	1406 ± 18		52
Parsley[b]	32.3–104.7	5.3–53	56.7–479.2	94.3–541.5	47, 58
					52
Parsnip[a]	0.01–10.5	170–682	213–430		58–61
Angelica[a]		427.3	3477.0		59
Heracleum[a]	6.1–6.5	140–150	64–68	220 ± 9	62

[a] Concentration expressed on a fresh weight basis.
[b] Concentration expressed on a dry weight basis.
[c] See Fig 2 for the chemical structure.

and the auto-analysis test is performed in test tubes pre-filled with a powdered, coliform-specific indicator nutrient.[76] After incubation at 35°C for 24h, any indicator-positive tube is illuminated with UVA, and fluorescence of the solution indicates the presence of *E coli* and hence a risk of faecal contamination. The bacterium *Pseudomonas aeruginosa* which causes rots in eggs, meat and fish can also be detected by its yellow–green fluorescence under UVA radiation.[76]

In another application a redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), has been employed for the direct epifluorescent microscopic enumeration of live bacteria in environmental samples.[78] The CTC competes directly with molecular oxygen as an electron acceptor, and the reducing power generated by the electron transport system converts CTC into its reduced formazan, which accumulates in metabolically active bacteria. When illuminated with long-wave UV (>350nm), the reduced CTC fluoresces bright red and is easily detected. However, the application of this technique to foods needs to be carefully assessed, as some foods may contain significant levels of natural or artificial quenchers.

In order to reduce the risk of microbial contamination from flying insects, much use is made of traps in which a UVA fluorescent lamp is mounted in a unit containing a high-voltage grid. The insect, attracted by the UVA lamp, flies into the unit and is electrocuted in the air gap between the high-voltage grid and a grounded metal screen. Such units are commonly found in areas where food is prepared and/or sold.[2]

CONCLUSIONS

While the UVA/furocoumarin system has a superficial attraction for sanitising solutions, ie better penetration of the radiation, it has yet to find a commercial niche. By contrast, UVC enjoys a good reputation for sanitising the air or food contact surfaces, and it seems likely that its use will expand as the supporting technology improves. For example, safe drinking water can assured by exposure to UVC systems so long as the associated filtration system is capable of removing all particulates, and recent advances in North America suggest that fluids containing suspended solids can be treated as well. The security offered by this latter system remains under scrutiny, for it is not clear at present whether the radiation levels would be effective if a sample of fruit juice, for example, was contaminated with *E coli* or some other pathogen prior to treatment.

Nevertheless, it is evident that the food industry is faced with two conflicting pressures. On the one hand, there is the need to produce microbiologically 'safe' food, while on the other, consumers are seeking foods with more natural flavours and textures. Consequently, a resurgence of interest in UVC could well be appropriate, for it does seem that UV radiation is one of the least exploited antimicrobial treatments for surfaces and, perhaps, foods themselves.

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ORIGINAL ARTICLE

The antimicrobial effects of long-wave ultra-violet light and furocoumarins on some micro-organisms that occur in cheese brines

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Listeria monocytogenes *and* Escherichia coli *O157:H7 have been reported to survive in the brines used to store cheeses like Feta or Teleme, but such brines cannot be heat sterilized as the yeasts and lactic acid bacteria essential for normal cheese maturation are present as well. Long-wave UV light (UVA ~ 365 nm), acting in conjunction with photosensitizing compounds (e.g. furocoumarins like psoralen) might have more limited microbiocidal properties, so that, perhaps, pathogens could be eliminated from the cheese brine but not the desired yeasts and lactic acid bateria. In laboratory trials, UVA (intensity 45 W m* $^{-2}$*, exposure time 60 s) with psoralen (5mg l* $^{-1}$*) was active against* Listeria innocua *— chosen to mimic the behaviour of* L. monocytogenes—Escherichia coli *O157:H7 and* Staphylococcus aureus *in a physiologically neutral solution, but* E. coli *O157:H7 (99% reduction in viable cell count) and* L. innocua *(99·8% reduction) were slightly less sensitive than* S. aureus *(99·99% reduction). Yeasts from Feta cheese brines were less affected by the same UVA/furocoumarin system —* Debaryomyces hansenii *(97·5% reduction) and* Yarrowia lipolytica *(82·7% reduction), as were typical lactic acid bacteria, namely* Lactobacillus paracasei *subsp.* paracasei *(97·8% reduction) and* Lactobacillus plantarum *(91·9% reduction). A UVA exposure time of 100 s with psoralen (5 mg l* $^{-1}$*) was lethal to the 'pathogens' but, against the desirable species, only* Yarrowia lipolytica *(97·4% reduction) readily survived the same treatment. It was concluded that the UVA/furocoumarin system was microbicidal but not, at least in the form under test, sufficiently selective in its action for use with cheese brines where certain of the microfloras need to be retained.*

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Introduction

In general, dairy products enjoy a good reputation with respect to public health but, even so, there have been a number of cases where cheeses have acted as carriers of foodborne infections (Keceli and Robinson 1997). One group of especial interest in this respect are the white brined cheeses, for not only are they often stored and consumed at temperatures > 20°C, but poorly maintained brine tanks can become ready sources of contamination. In particular, Gohil et al. (1995), Katic (1995), Larson et al. (1999), Papageorgiou and Marth (1989) and

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Ramsaran et al. (1998) have all highlighted the point that important pathogens like *Escherichia coli* O157:H7 and *Listeria monocytogenes* can survive in the brines used to store Feta or similar cheeses.

Obviously techniques like pasteurization or microfiltration could be employed to improve the microbiological quality of brines (Otero-Rodriguez et al. 1998, Ottosen and Konigsfeldt 1999), but such decontamination procedures are seldom applied to the brines for Feta and related cheeses (Zerfiridis 1994). This reluctance to 'sterilize' cheese brines stems from the fact that such processes will reduce also the total counts of lactic acid bacteria (LAB) and yeasts, and there is good evidence that both groups play an essential role in the development of the sensory properties desired in brined cheeses (Litopoulou-Tzanetaki 1984, Litopoulou-Tzanetaki et al. 1993).

What is required, therefore, is a procedure that will eliminate pathogens from brine while leaving the desirable microfloras intact, and one such approach could involve the irradiation of thin films of brine with ultra-violet (UV) light. Irradiation with short-wave length UV ($\sim$254 nm) has been considered, but it has two disadvantages in the present context, namely penetration into liquids is limited and the germicidal impact is non-selective (Bintsis et al. 2000). However, long-wave UV (UVA), i.e. wave-lengths in the region of 320 - 400 nm, can penetrate further into water (Peak et al. 1981) and, because the lethality of UVA against micro-organisms is low compared with short-wave UV light, inter-generic selectivity could become a possibility.

UVA affects microbial cells by causing membrane damage, or by inducing the generation of active oxygen species or H_2O_2; either of the latter may be the primary agents of cell damage (Moss and Smith 1981, Kramer and Ames 1987). However, such effects have little impact on microbial cells unless the lethality can be enhanced by the coincidental absorption into the cells of an exogenous photosensitizer (Mitchell 1978). One group of compounds that meets this requirement are the tricyclic furocoumarins which are formed by the fusion of a furan ring with a coumarin molecule (Cimino et al. 1985). The molecular mechanism of the photodestruction of microbial cells is believed to involve: the formation of a complex with DNA via the intercalation of furocoumarins between adjacent bases; photobinding of the furocoumarins to the pyrimidine bases; and cross-linking between furocoumarins located in different strands of DNA (Cimino et al. 1985).

Once cross-links have formed, cell division ceases and, unless the repair mechanisms are operative, death of the cell follows (Scott et al. 1976).

Five plant families, namely the *Umbelliferae* (e.g. celery, parsley and parsnip), *Rutaceae* (e.g. bergamot fruit and lime), *Moraceae* (e.g. fig), *Leguminosae* and the *Orchidaceae* have been identified as natural sources of furocoumarins, and dried plant materials, expressed juice or even pure compounds like psoralen could be considered for direct addition to a brine prior to irradiation with UVA. However, as the natural levels of furocoumarins in plants are low, large volumes of harvested crops would have to be handled if fresh or dried materials were to be added to a brine. In practice, this approach is likely to be unacceptable because: (a) moulds or other undesirable contaminants could be introduced into the cheese factory; and (b) routine skin contact with natural plant materials containing furocoumarins needs to be controlled to avoid the development of allergic reactions (Bethea et al. 1999).

The use of concentrated plant extracts or purified furocoumarins seems, therefore, to offer the most likely route, the more so as control of the concentration of furocoumarin in the brine would be much easier. Apart from any practical considerations, this latter point is important from a public health standpoint for, although the anticipated dose rates of furocoumarin(s) in the brine are very low (see later), there are toxicological risks for humans if the daily intake of psoralen, for example, exceeds 20 mg (IARC 1983, Stern et al. 1984). However, as intakes for consumers of brined cheese would be unlikely to exceed even a few micrograms per day, the risk of adverse reactions would appear to be negligible. Consequently, it was decided that the potential of the UVA/furocoumarin system to selectively eliminate pathogens like *E. coli* O157:H7 and *L. monocytogenes* from cheese brine merited examination

and, in particular, the aims of this present study were to: (a) measure the killing power of UVA, in conjunction with pure psoralen, against some selected pathogens; and (b) compare the activity of the same system against a number of benign micro-organisms isolated from Feta cheese brine.

Materials and Methods

Selection of the cultures

As the ecology of *Listeria innocua* (NCTC 11288) is similar to *L. monocytogenes* and the species has been reported to survive in cans of Feta cheese (Gohil et al. 1995, Papageorgiou and Marth 1989), this non-pathogenic species was used to simulate the behaviour of *L. monocytogenes*. In addition, both *E. coli* and *Staphylococcus aureus* have been cited as potential post-pasteurization contaminants of Feta cheese (Erkmen 1995, Ramsaran et al. 1998), so that one strain of *E. coli* O157:H7 (PHLS-E19475: VT–) and one of *S. aureus* (NCTC 1803) were included in the trials.

As retention of the LAB and yeasts that are typical of storage brines for cheese was regarded as essential, a number of yeasts and LAB were isolated from samples of Feta cheese brine from Greece. The cultures included *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Lactobacillus paracasei*, subsp. *paracasei* and *Lactobacillus plantarum*, and the UVA/furocoumarin system was tested against each of the four organisms.

The two species of yeast were maintained on slopes of Yeast Extract Dextrose Agar (Kreger van Rij 1984), while the species of *Lactobacillus* were maintained on slopes of MRS Agar (Oxoid Code No: CM361, Unipath Ltd., Basingstoke, Hampshire, UK.) Tryptone Soya Agar [Tryptone Soya Broth (Oxoid Code No: CM129)+1·5% Agar] was used for maintenance of *L. innocua*, *E. coli* O157:H7 and *S. aureus*. In every case, the freshly inoculated slopes were incubated at 30°C until growth was visible to the naked eye, and the cultures were then stored at 5°C ± 1°C. Each stock culture was subcultured either prior to use or monthly.

Preparation of bacterial suspensions

It has been suggested that stationary phase cells are much less sensitive than exponential phase cells to photosensitization (Oginsky et al. 1959, Griego et al. 1981), and hence it was decided that the UVA/psoralen system should be tested against cells in the stationary phase of growth. Growth curves for the 'pathogens' were obtained by inoculating loopfuls of *L. innocua*, *E. coli* O157:H7 or *S. aureus* from a stock culture (see above) into sterile Tryptone Soya Broth (duplicate lots of 50 ml in 100 ml flasks), followed by incubation at 30°C. At 2-h intervals, the flasks were shaken and duplicate 1 ml amounts of broth were removed and added to universal bottles containing sterile Maximum Recovery Diluent (MRD — Oxoid Code No: CM733; 9 ml). At time zero, the cell counts were obtained by direct plating (pour plates) of duplicate aliquots (1 ml) of this initial dilution in Tryptone Soya Agar, but further dilutions were made as the cell counts increased with time. After 12 h, another set of duplicate flasks was inoculated for each culture, and the plating procedure repeated beginning at 14 h and continued until 24 h.

A similar procedure was repeated for the yeast cultures in Yeast Dextrose Broth (Kreger van Rij 1984) at 30°C, but in this case the flasks were sequenced so that the plating procedures began at 0, 14 and 28 h with cessation after 48 h. The counts for lactobacilli were obtained in the manner employed for the 'pathogens', but in MRS Broth (Oxoid Code No: CM359) at 30°C. The onset of the stationary phase was calculated as 10–14 h from the time of inoculation for *L. innocua, E. coli* O157:H7 and *S. aureus*, 12–16 h for the lactobacilli and 40–46 h for the species of yeast. As a routine, therefore, 16-h old cultures of *L. innocua*, *E. coli* O157:H7 and *S. aureus* were used to ensure that the bacteria were in the stationary phase, and 18-h cultures for the lactobacilli and 48-h cultures for tests involving the yeasts.

Preparation of the standard solution of psoralen

To prepare the standard solution of psoralen, 10 mg psoralen (Sigma Ltd., Poole, Dorset, UK) were dissolved in 2 ml of HPLC grade

ethanol (BDH, Poole, Dorset, UK) and 98 ml sterile distilled water were added; this solution (pH 6·5) was stored at 4°C. This concentration was selected as each ml of stock solution contained 0·1 mg of psoralen so that, when 1 ml was added to 9 ml to test bacterial/yeast suspension, the final concentration was equivalent to 10 mg l^{-1}. This value is double that found by Lin et al. (1994) to be effective against pathogens, and was the maximum concentration tested in this present study.

Sources of UVA

A Black-Ray Display Lamp (365 nm, 15 W) (Ultra-Violet Products, Cambridge, UK) was used for most of the tests, and the intensity, at a distance of 10 cm, was 45 W m^{-2}. During many of the experiments, the intensity of the lamp was monitored, and the variation never exceeded 5%.

In addition, the effect of a 100 watt lamp on the lethality of the UVA/furocoumarin system was examined, and the intensity of the B-100AP lamp was reported to be 150 W m^{-2} at 15 cm (Ultra-Violet Products).

Protocol for UV/psoralen treatment

The test cultures were obtained by growing *L. innocua*, *E. coli* O157:H7 and *S. aureus* in Tryptone Soya Broth (15 ml) at 30°C for 16 h, the yeast cultures in Yeast Dextrose Broth at 30°C for 48 h and the lactobacilli in MRS Broth at 30°C for 18 h. Then, for each organism in turn, the culture was agitated with a Whirlimix (Fisher Scientific, Loughborough, Leicestershire, UK) and the entire content transferred to a sterile centrifuge tube. The cells were harvested by centrifugation at 4,000 *g* for 10 min at ambient temperature in a IEC Centra-4X centrifuge and, after decanting the original broth, the cells were resuspended in sterile MRD at 20°C. This suspension was then diluted, based on the results from some preliminary tests, to give a working solution containing $\sim 10^5$ cfu ml^{-1}; confirmation was obtained in every case by means of total colony counts at time zero (see later).

Volumes of 9·0–9·9 ml of a selected suspension containing $\sim 10^5$ cfu ml^{-1} of a bacterium or yeast were pipetted into a sterile beaker (100 ml), and mixed with 0·1–1 ml amounts of the stock solution of psoralen to give 10 ml in total; the addition of 1 ml of the psoralen solution gave a final concentration of 10 mg psoralen l^{-1}, and 0·1 ml of the standard solution gave a final concentration of psoralen of 1 mg l^{-1}. The depth of the suspension/psoralen mixture was approximately 1 cm, and the concentrations of psoralen used were 1, 3, 5, 7 and 10 mg l^{-1}.

Each mixture was left for 10 min for some of the psoralen to enter the cells, and the beaker was then placed under the UVA lamp; previous experiments (data not shown) indicated that cells subject to equilibration times for longer periods (up 60 min) were not more susceptible to UVA radiation. The distance between the lamp and the surface of the liquid under test was 10 cm.

The number of surviving micro-organisms was determined at intervals of 20 s by withdrawing 0·1 ml amounts from the suspension with a sterile Gilson pipette (Anachem Ltd., Luton, Bedfordshire, UK) and preparing serial dilutions in MRD. However, after the period of 60 s for the 'pathogens' and 80 s for the lactobacilli and yeasts, the numbers of cells had declined sufficiently for the 0·1 ml amounts to be pipetted directly onto the surfaces of duplicate, pre-poured plates of Yeast Dextrose Agar, MRS Agar or Tryptone Soya Agar — depending on the culture. For the less irradiated samples and the 'control' (zero time), 0·1 ml amounts from dilutions 10^{-1}–10^{-3} — prepared after the last extraction at 120 s — were spread onto duplicate, pre-poured plates of the appropriate medium. On completion of the counts at 30°C, survival of the cells was calculated in order to evaluate the anti-microbial effect of specific time/concentration applications of the furocoumarin/UVA system. The percentage of organisms surviving at a given time was calculated from $n/no. \times 100$, where n = number of organisms (cfu ml^{-1}) recovered at time t, and no = initial number of micro-organism (cfu ml^{-1}) in the suspension.

Controls

In order to determine whether either UVA or psoralen alone had an effect on the survival of

the micro-organisms, two control treatments were carried out: UVA illumination at intensities of 45 and 150 W m^{-2} without the addition of psoralen, and the addition of psoralen (5 mg l^{-1}) without exposure to UVA.

Results and Discussion

Effect of different concentrations of psoralen

The bactericidal effect of different concentrations of psoralen plus UVA illumination (45 W m^{-2}) was studied over a period of 2 min against the bacterial pathogens and *L. innocua*, and the results are shown in Table 1. It can be seen that, in the presence of psoralen at a concentration of 5 mg l^{-1}, an acceptable inactivation rate for *L. innocua*, *S. aureus* and *E. coli* O157:H7 was obtained over 60 s, i.e. the reductions in viable counts were 99·8% for *L. innocua*, 99·99% for *S. aureus* and 99·0% for *E. coli* O157:H7. Not unexpectedly, a concentration of 10 mg l^{-1} increased the lethality of the system but, as there would be good public health reasons for using the minimum practical concentration of psoralen in a commercial system, a concentration of 5 mg l^{-1} was selected as providing a feasible option. Furthermore, it is evident that none of the 'pathogens' could be detected with an increase in exposure time to 100 s, and any practical application of this technique could take this point into account.

Overall, these results were broadly in agreement with the reports of Oginski et al. (1959), Manderfeld et al. (1997), Lin et al. (1994) and Ulate-Rodriguez et al. (1997) who found that some Gram-positive bacteria — *S. aureus* is the example from this study — can be more easily inactivated by furocoumarins/UVA than Gram-negative species. This comparative resistance of Gram-negative bacteria to the

Table 1. Inactivation of suspensions of *L. innocua*, *E. coli* O157:H7 and *S. aureus* in MRD using different concentrations of psoralen between 1–10 mg l^{-1} and exposed to UVA (45 W m^{-2}) irradiation

	1	3	5	7.	10
			L. innocua		
Time/concentration of psoralen (mg l^{-1})					
0	100	100	100	100	100
20	85	82	50	44	40
40	58	58	16	5	2
60	36	16	0·2	0·05	< 0·01
80	29	0·8	0·04	< 0·01	ND
100	24	0·02	ND	ND	ND
			S. aureus		
0	100	100	100	100	100
20	77	89	39	16	6
40	33	38	0·06	0·02	0·01
60	6	0·05	0·01	ND	ND
80	2·8	ND	ND	ND	ND
100	0·7	ND	ND	ND	ND
			E. coli O157		
0	100	100	100	100	100
20	67	53	50	11	3
40	50	31	18	0·2	0·2
60	32	5	1	0·07	0·04
80	7	0·07	0·03	ND	ND
100	0·5	ND	ND	ND	ND

ND = Not detected.
All figures as surviving fraction (%) of the original cell counts.

Table 2. Inactivation of *Lb. paracasei* subsp. *paracasei*, *Lb. plantarum*, *D. hansenii* and *Y. lipolytica* in MRD dosed with 5 mg l^{-1} of psoralen and exposed to UVA (45 W m^{-2}) irradiation

Time (s)	*Lb. paracasei* subsp. *paracasei*	*Lb. plantarum*	*D. hansenii*	*Y. lipolytica*
0	100	100	100	100
20	75·5	94·1	43·6	31·1
40	24·5	70·6	14·5	24·4
60	2·2	6·1	2·5	17·3
80	0·05	0·06	1·3	8·2
100	ND	0·008	0·9	2·6
120	ND	ND	0·6	2·2

ND = Not detected.
All figures as surviving fraction (%) of the original cell counts.

penetration of hydrophobic compounds (e.g. furocoumarins) may be a reflection of the fact that the outer cell membrane contains lipopolysaccharides which can delay, or perhaps prevent, the entry of such molecules into the cell and reaction with the DNA (Freese et al. 1973). It was notable also that some cells of *L. innocua* appeared to be more resistant to the system than the Gram-negative species, so that clearly generalisations about the reactions of Gram-negative/Gram-positive genera must be somewhat tentative.

When some micro-organisms that constitute the natural micro-flora of Feta cheese brine were exposed to UVA and psoralen at a concentration of 5 mg l^{-1} (see Table 2), it was notable that, while the yeasts showed initial sensitivity to the treatment, several thousand cells survived exposure for 100 s — the exposure time necessary to eradicate the 'pathogens' under the same conditions. The isolate of *Lb. plantarum* was slightly less susceptible than the Gram-positive 'pathogens', but *Lb. paracasei* subsp. *paracasei* was no longer detectable after 100 s. The contrasted degrees of inhibition shown by the yeasts and lactobacilli may be due to differences in either their cell membrane structures or repair processes, or to differences in the distribution of the furocoumarins within the cell and/or interactions with other components, such as proteins and/or fatty acids (Murray et al. 1982, Sastry 1997, Specht et al. 1988). In addition, the photochemistry of molecules other than nucleic acids becomes of great importance in eucaryotic cells, since the nuclei in such cells are shielded by UV absorbing material in the surrounding cytoplasm (Smith and Hanawalk 1969).

High intensity lamp

Although the use of the lower intensity lamp might be advantageous in ensuring the survival of the yeast cells, the system would become more secure if total lethality against the Gram-negative bacteria and *L. innocua* could be attained. To this end, the use of a high intensity (150 W m^{-2} UVA) lamp was studied with psoralen at a concentration of 5 mg l^{-1}. The results are shown in Fig. 1, and again *S. aureus* was most susceptible to the synergistic action of psoralen/UVA. *Escherichia coli* O157:H7 was also severely inactivated by the 100 W lamp, and a 60-s treatment

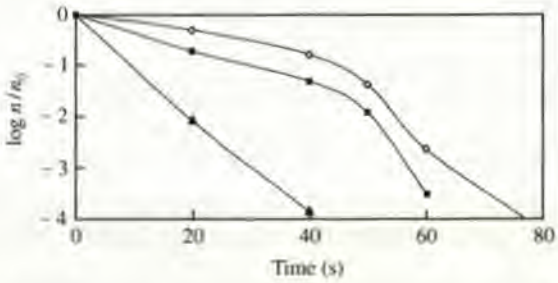

Figure 1. Inactivation of *L. innocua* (—◇—) *E. coli* O157:H7 (—■—) and *S. aureus* (—▲—) in MRD dosed with 5 mg l^{-1} psoralen and exposed to UVA (150 W m^{-2}) irradiation. Data reported as log $n/no.$, where n = number of organisms (cfu ml^{-1}) recovered at time t, and $no.$ = initial number of micro-organism (cfu ml^{-1}) in the suspension.

resulted in a 99·97% reduction in the viable count. It has been reported that large dosages of UVA increase the permeability of cells to various substances (Giese 1997), and the same effect may have caused the increased susceptibility of the cells of *E. coli* O157:H7 to the psoralen/UVA system. In a separate test (data not shown), cell counts of the beneficial cultures, namely *D. hansenii*, *Y. lipolytica*, *Lb. paracasei* subsp. *paracasei* and *Lb. plantarum* showed the same pattern of inactivation as shown in Table 2, except that inactivation was more rapid.

Clearly the higher intensity UVA was more microbicidal but whether, in a practical situation, the timing could be controlled with sufficient accuracy to make the system selective in favour of yeasts and lactobacilli would require further study.

Experimental controls

In order to ensure that both UVA radiation and the photosensitiser were essential for inactivation of the micro-organisms, a final trial examined the effects of psoralen at $5\,mg\,l^{-1}$ in the absence of UVA radiation, and UVA radiation alone at intensities of 45 and $150\,W\,m^{-2}$ for 120 s. The results are shown in Table 3.

The treatment with psoralen ($5\,mg\,l^{-1}$) alone proved that furocoumarins require the synergistic action of UVA to inhibit microbial cells and, moreover, that the ethanol used to dissolve the psoralen had no detectable effect on the micro-organisms. Similarly, UVA alone at $45\,W\,m^{-2}$ had little effect on the survival of the micro-organisms, at least over the exposure time used, and even the higher intensity lamp only achieved a reduction of around 20% with the Gram-positive species. Yousef and Marth (1988) found that irradiation of cells of *Listeria* spp. with UVA for up to 10 min did not result in any significant decrease in the counts, but Kramer and Ames (1987) postulated that, while microbial cells may be resistant to short exposures to UVA radiation, they will begin to die rapidly after treatment for 3–4 h.

Conclusion

The above results suggest that a furocoumarin/UVA system could destroy those pathogenic bacteria which are considered to be the major risks associated Feta and other white-brined cheeses. Some of the species of yeast that are essential components of the natural microfloras of Feta cheese brines seemed to be more resistant, but the counts of *Lb. paracasei* subsp. *paracasei* and *Lb. plantarum* were seriously depleted under the furocoumarin/UVA regime needed to eliminate pathogens. Although the exact role(s) of yeasts and lactobacilli in the maturation of Feta cheese is not known in detail, it is likely that the reduction in numbers observed in these present tests could prove unacceptable. However, if the furocoumarin/UVA could be made selective by, perhaps, using one of the new synthetic furocoumarins that are more photoactive than natural psoralen (Dalla-Via et al. 1999, Lin et al. 1997, MargolisNunno et al. 1997, Santana et al. 2000), then consideration would need to be given also to the fact that cells exposed to harsh, but full nutrient, environments like cheese brines, can invoke protective mechanisms against a variety of inhibitory treatments (Raja et al. 1991). For example, acid-adapted cells of *L. monocytogenes* were found to be more resistant to bacteriocins than normal cells (van Schaik et al. 1999), or showed enhanced survival in milk fermented by cultures

Table 3. Surviving fraction (%) of the organisms indicated from suspensions in MRD exposed to UVA at the intensities indicated or psoralen without UVA

Treatment	*L. innocua*	*E. coli*	*S. aureus*	*Lb. paracasei* subsp. *paracasei*	*Y. lipolytica*
Psoralen[1]	96	92	92	92	99
$45\,W\,m^{-2}$	92	100	92	90	100
$150\,W\,m^{-2}$	83	93	84	79	99

[1]Concentration of psoralen was $5\,mg\,l^{-1}$.
All results were for exposures of 120 s.

of lactic acid bacteria (Gahan et al. 1996). Similarly, cells of *E. coli* incubated at a mildly acid pH (pH 5) showed an increase in UV resistance compared with cells grown at pH 7 (Goodson and Rowbury 1991).

Overall, this study has confirmed that the UVA/psoralen treatment does have potential as a novel decontamination process for liquid products but, at present, the high cost of the pure psoralen, together with its toxicity at high concentrations, may prevent the system from being widely applied. However, if one of the synthetic furocoumarins proves to be less expensive, less toxic to humans and selective in its action—lethal to bacteria, for example, under UVA but not yeasts, then the use of the system to decontaminate cheese brines could become a reality.

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Bafut: a Cameroon cheese

A study of the manufacture and characteristics of Bafut, a local cheese produced at a convent in Cameroon.

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In the Cameroon, milk production is comparatively undeveloped, and most of the available cow's milk is produced by the Fulani cattlemen from the Adamaoua Plateau or the north-west regions of the country. The system of farming is still semi-nomadic, with the cattle being held in the vicinity of the village during the wet season, and then taken to lower pastures during the hot months.

Milking is all done by hand, and any milk not required by the owners is either boiled and sold as liquid milk, or allowed to sour naturally to provide a base for a sorghum or maize porridge. However, the Fulani cattle do not give high yields (7,13), and hence the volume of milk placed on the market tends to be limited.

More recently, attempts have been made to introduce cattle capable of providing higher and more consistent yields of milk, either alone or after crossing with native cattle, and one such herd was sited at Wum, Cameroon (5).

As a result, fresh milk became available for processing into cheese, for it was considered that a hard/semi-hard variety might both withstand the rigours of sale at the local market, and bring a valuable source of nutrients to the urban population. From this tentative start, cheese production has spread to a number of locations in the north-west, including the village of Bafut.

Today, Bafut cheese has come to be recognised locally as a hard, cylindrical cheese of around 2kg in weight, and covered with a dry, hard rind formed by moisture loss during maturation.

However, in spite of its availability on the market, no attempt has been made to standardise the product, and hence the aim of this present study to (i) document a typical procedure of local manufacture; and (ii) analyse some typical examples of the finished cheese, both chemically and microbiologically, so as to obtain an indication of the characteristics of Bafut cheese.

With this information, it might be possible to standardise both the process of manufacture and the end product, so opening the way for possibly expanding production from either fresh, recombined or even filled milks.

Figure 1. The mixed herd of cattle that provides the milk for Bafut cheese

Although Bafut cheese is produced at a number of locations, one operation capable of manufacturing cheese of consistent quality is located at the Convent of the Sisters of St Emmanuel at Bafut, and it was the essential features of this particular process that were recorded.

Normal manufacturing methods

Fresh morning milk is taken from a mixed herd of Jersey, Jersey X White Fulani and Boran X White Fulani cows (see figure 1), and this milk is mixed with an equal quantity of skimmed milk, ie separated milk from the previous evening. After warming the mixed milks to 35°C, around 0.5g of rennet powder/10 litres of milk is suspended in water (4-500 ml), and then added to the vat. Stirring for 2-5 minutes ensures an even distribution of the rennet, and the milk is then allowed to stand for 40-50 minutes for a firm coagulum to form.

The gel is then cut with a knife into coarse cubes, and the mass is left undisturbed for 15-20 minutes for the initial separation of the whey and additional acid development. The natural microflora of the raw milk provides the source of lactic acid, and the overnight storage of the evening milk may encourage the build-up of a desirable population of lactococci and/or lactobacilli. The pieces of curd are then further reduced in size and allowed to settle to the bottom of the vat. After a further rest period, the surface whey is drawn off until about 4-5 litres remain, and salt is added to give a level in the finished cheese of around 1.5%.

A holding period of 3-5 minutes allows the salt to become evenly distributed, and the curd is then ladled into a basket lined with cheese cloth. The cloth is drawn around the curd to form a 'ball', and manual pressure encourages the curd to coalesce with the expulsion of any residual whey. When the cheese no longer sticks to the cloth, the cloth is removed and the cheese is placed back in the basket; the final shaping of the cheese takes place at this point.

The cheeses, still in their individual baskets, are then transferred to a well-ventilated maturation room (17-20°C). Daily turning within the basket takes place until the surfaces of the cheeses are thoroughly dry, at

Table 1. Mean values obtained by the analysis of 12 samples of Bafut cheese.

	Mean	Range
Fat	25.0	17.5 - 29.5
Protein	26.1	22.2 - 33.6
Moisture	41.6	40.3 - 45.1
Fat-in-Dry-Matter	42.8	38.5 - 49.5
Titratable Acidity	2.1	1.8 - 2.6
pH	4.3	3.9 - 4.6
Total Volatile Substances	0.09	0.05 - 0.15
Free Fatty Acids	0.3	0.18 - 0.33
Salt	1.3	1.01 - 1.53
Tyrosine (mg/5ml filtrate)	0.24	0.16 - 0.30

All figures as g/100 g of cheese as consumed, except for the index of proteolysis. The figures for Volatile Substances and Free Fatty Acids have been reported as "% lactic acid" as recommended by Kosikowski (1982).

which point they are placed on a wooden shelf to complete their maturation over a period of 4-6 weeks (see figure 2). Regular turning and washing with saturated brine is essential to prevent the growth of adventitious moulds on the surface, but the brine may well encourage the development of yeasts and bacteria, including *Brevibacterium linens*; it is likely that this latter microflora contributes to the flavour of the retail cheese.

Figure 2. The cheese is stored at ambient temperature for one month to achieve the desired flavour

Characteristics of Bafut cheese

Twelve cheeses were obtained from the Convent of the Sisters of Emmanuel at the end of four weeks maturation, and each was subjected to the following chemical analyses:

- Moisture content: determined by the method described in (9);
- Crude protein: estimated by the Kjeldahl method as suggested by the AOAC (2);
- Fat: determined by the Gerber procedure (9);
- Titratable acidity: measured by titration against N/9 NaOH with phenolphthalein as the indicator (12);
- Volatile substances: the level of total volatile substances was determined by the method described in (4), and that of free fatty acids by the distillation procedure of Dulley and Grieve (3);
- Salt content: determined by the procedure described in (10);
- Tyrosine index: this measure of proteolysis was based on the method of Hull (6).

The same cheeses were subjected to appropriate microbiological assessments (9) by taking 20g sub-samples and macerating them in 180ml of sodium citrate (2%); dilution series were prepared in one quarter strength Ringer's solutions (9ml). Total colony counts and aerobic spore counts were obtained by the pour plate method (30°C) employing Milk Agar (11), while counts for the *coli-aerogenes* group were made on violet red bile agar.

Tubes of brilliant green bile broth were inoculated with colonies selected from the latter plates, and incubated at 44°C along with tubes of peptone water. Gas production in the broth and an indole-positive reaction in peptone water was taken as confirmation for *Escherichia coli*. The pour plate procedure was employed also for total yeast and mould counts (rose Bengal chloramphenicol agar) and Lactobacilli (MRS agar), and surface spread plates (Baird Parker Medium) were prepared to estimate the numbers of coagulase-positive staphylococci present (11). Practical constraints meant that it was not possible to confirm that the suspect colonies were coagulase-positive.

The mean values for the principal components, namely fat, protein and moisture, were found to be not unlike many semi-hard cheeses, such as Edam or Gouda (see Table 1), while the wide variations in composition are merely a confirmation that the basic process lacks certain essential controls. The mean pH is, of course, much lower that would be found with many European cheeses but, given the avenues for microbial contamination of Bafut cheese, the element of inhibition offered by the elevated acidity may not be unwelcome.

The high ambient temperatures of storage are conducive to enzymatic activity, and the level of free fatty acids, together with the degree of proteolysis, confirms that the biochemical reactions associated with maturation have been fairly rapid. The strong flavour that results is appreciated by local consumers, but the levels of salt recorded for these particular samples were much lower than those found in many African cheeses (1).

The poor microbiological quality of the cheese compared with similar products from Europe is a reflection of the use of raw milk, and milk that has been held overnight at ambient temperature. The high counts of the 'coli-aerogenes' group, in particular, suggests that the cheese 'as consumed' contains appreciable numbers of *Escherichia coli* (see Table 2). However, it would appear that this aspect of quality could be controlled without too much difficulty, for at least one of the cheeses sampled gave counts of <10 cfu/g for both *Staphylococcus* sp and genera in the 'coli-aerogenes' group.

Despite some concerns over microbial quality and compositional variability, it is important that (a) a form of cheese is being produced, on a routine basis, in an area where cheese was previously little known; and (b) the product appears to be popular with consumers. Obviously, there is a need to further standardise the process before expansion of production could be contemplated, but the Sisters of St Emmanual deserve every credit for creating a sound basis for further development.

Table 2. Some components of the microflora of Bafut cheese at four weeks from manufacture.

Total Colony Count	$21,300 \times 10^6$
Aerobic Spore Count	47,500
Coli-aerogenes Group	6,218
Escherichia coli (% cheeses testing positive)	42%
Yeasts & Moulds	1,583
Staphylococcus sp (coagulase-positive)	608
Total Lactobacilli	$1,400 \times 10^6$

Colony counts (means of 12 individual cheeses) expressed as colony-forming units (cfu)/g of cheese.

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PAPER

Preservation of raw milk by activation of the natural lactoperoxidase systems

M.S. Haddadin,[†] S.A. Ibrahim[†] and R.K. Robinson[‡*]

The preservation of raw ovine, bovine and caprine milks by activation of their natural lactoperoxidase (LP) systems was investigated. Milk samples with different concentrations of thiocyanate ions (SCN^-) ranging from 15 to 150 mg/l and hydrogen peroxide (H_2O_2) ranging from 10 to 100 mg/l were stored at 4°, 22° and 30°C. Changes in titratable acidity, total colony counts and coliform counts of the milk samples were followed during storage. No significant differences were observed between samples with different concentrations of the added reagents, and this indicated that concentrations of 15 mg/l (SCN^-) and 10 mg/l (H_2O_2) would be adequate for preserving milks of different mammals – at least at 4°C. At higher temperatures, the effectiveness of the LP systems was, as monitored by the increases in acidity, of much shorter duration. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

The lactoperoxidase (LP) system is a naturally occurring, antimicrobial mechanism found in raw milk. It can exert a bacteriostatic effect on both Gram-positive and Gram-negative bacteria, including those psychrotrophic bacteria which decrease the shelf-life of liquid milk at 4°C (Bjorck, 1978; Marshall *et al.*, 1986; Kamau *et al.*, 1990; Wolfson and Sumner, 1994). The preservative action of the LP system in bovine milk has been well established (Reiter *et al.*, 1976), and Pruitt and Reiter (1985) reported that activation of the system depends on the concentrations of two reactants, thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2). In particular, the LP system has the ability to catalyse the oxidation of thiocyanate by hydrogen peroxide with the production of the antibacterial hypothiocyanite ($OSCN^-$) and other intermediates (Modi *et al.*, 1991). These compounds, which can be further oxidized to end-products that are harmless to humans, have the ability to reduce bacterial growth by damaging the cell membranes and inhibiting the activity of many cytoplasmic enzymes.

The normal concentrations of lactoperoxidase in bovine and ovine milks are reported to be above the minimum necessary for antibacterial activity, and hence the limiting factors are thiocyanate and hydrogen peroxide (Bjorck, 1978; Medina *et al.*, 1989). In some circumstances, thiocyanate is present in bovine milk at levels sufficient to support an antimicrobial effect, eg up to 15 mg/l (Reiter, 1985) but, in ovine milk, the level can decline to 0.4 mg/l (Medina *et al.*, 1989). Therefore, if the concentrations of SCN^- and H_2O_2 could be standardized, the milk of the important domestic mammals should show enhanced shelf-life even, perhaps, at ambient temperatures. However, while there are some reports on the effect of different concentrations of sodium thiocyanate/hydrogen peroxide on the activity of the

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LP system at different temperatures (Ewais *et al.*, 1985), most of these studies have dealt with bovine milk.

The purpose of this present study was, therefore, to evaluate the ability of the LP system to extend the shelf-life of ovine and caprine milks at refrigerated and ambient temperatures, using different concentrations of thiocyanate ions/hydrogen peroxide. The behaviour of bovine milk with the same levels of reactants was included for purposes of comparison.

MATERIALS AND METHODS

The experiment was repeated four times (each in duplicate), and samples of fresh ovine, caprine and bovine milk were collected as required from the University of Jordan farm. Each bulk sample was kept on ice during transport to the laboratory, and was always used within 24 h of collection. More rapid usage of the milks would have been desirable, but logistical problems meant that some time delay was inevitable; in any event, the storage period would have only served to lower the bacteriological quality of milk.

At the start of each experiment, the three milks were divided into portions (450 ml) in sterile, screw-cap, *Duran* bottles. The LP system of the samples was then activated by adding different amounts of sodium thiocyanate (Fisons, Loughborough, Leicestershire, UK) and sodium percarbonate (Fluka Chemie AG, CH-9470, Switzerland) – the latter acted as a source of hydrogen peroxide – to give four different concentrations of SCN^- and H_2O_2 in the ratios of 15:10, 75:50, 125:75 and 150:100. The control samples of raw milk received no treatment.

After the addition of the different amounts of sodium thiocyanate/percarbonate, the samples were thoroughly mixed and duplicate bottles were stored at each of three temperatures. Samples at simulated ambient temperatures (30°C or room temperature, 22°C) were incubated in water baths, and a further pair of samples was kept in a refrigerator at 4°C. After 0, 3, 6, 9, 12, 15 and 18 h at 30°C or room temperature (22°C), portions (20 ml) were withdrawn from each bottle of milk for analysis; from samples stored at 4°C, portions were withdrawn once every 24 h over a period of 6 days.

Bacteriological and chemical analyses

For measurement of the titratable acidity, a portion (10 ml) of each sample was mixed with 10 ml of distilled water and titrated with sodium hydroxide (N/9) to an end-point of pH 8.5 using an analytical-grade pH meter (Hana Instrument Model HI 8416, Limena, Italy). The volume of sodium hydroxide dispensed was divided by ten to give the titratable acidity as percentage lactic acid.

The bacteriological analyses were applied only to batches stored at 4°C, and these milks were tested for total colony counts and coliform counts on receipt from the farm, and then over six days storage. Duplicate sub-samples (1 ml) of the three milks were diluted in a series of peptone solutions (9 ml, 0.1%) down to 10^{-8}, and 1 ml aliquots from the appropriate dilutions (10^{-2}–10^{-4} for coliforms and 10^{-5}–10^{-7} for the total counts) were transferred to Petri dishes (9 cm). Yeast milk agar (Unipath Ltd, Basingstoke, UK) was employed for the total colony counts as recommended by BSI (1968), and the plates were incubated at 30°C for 72 hours; coliforms were enumerated on Violet Red Bile Agar (Unipath Ltd, Basingstoke, UK) after incubation at 37°C for 48 hours.

Statistical analysis

All data were analysed using the General Linear Models of the Statistical Analysis System (SAS, 1988) to determine the significance between treatment means.

RESULTS

When the different milks were stored at 4°C, the acidities of the treated samples of bovine milk were almost unchanged over four days ($P < 0.01$) and, even after six days, the increases were, at least in practical terms, negligible (see *Table 1*). Thus, it is widely accepted that the maximum acidity in bovine milk for cheesemaking, for example, should be 0.16% lactic

Table 1 Effect of different concentrations of SCN^- and H_2O_2 (mg/l) on the developed acidity (% lactic acid) of ovine, bovine and caprine milks stored at 4°C for up to 6 days

Time/ratios	Control			15:10			75:50			150:100*		
(d)	(O)	(B)	(C)	(O)	(B)	(C)	(O)	(B)	(C)	(O)	(B)	(C)
0	0.18	0.14	0.13	0.17	0.14	0.12	0.17	0.13	0.12	0.17	0.12	0.12
1	0.20	0.14	0.14	0.17	0.14	0.11	0.18	0.14	0.12	0.17	0.12	0.12
2	0.22	0.16	0.15	0.18	0.14	0.11	0.20	0.14	0.12	0.18	0.12	0.12
3	0.25	0.19	0.17	0.20	0.14	0.15	0.20	0.14	0.13	0.20	0.13	0.13
4	0.29	0.22	0.20	0.22	0.15	0.17	0.22	0.15	0.14	0.22	0.13	0.15
5	0.30	0.24	0.28	0.23	0.15	0.19	0.22	0.16	0.16	0.22	0.13	0.16
6	0.40	0.30	0.33	0.26	0.16	0.24	0.27	0.16	0.18	0.22	0.14	0.19

(O) = ovine milk; (B) = bovine milk; (C) = caprine milk.
*The results for 125:75 were similar.
Least square means: standard error = ±0.05, replications = 4.

acid (Tamime, 1993), so that even the lowest concentrations of thiocyanate and hydrogen peroxide were sufficient to keep the milk in an acceptable state for processing for six days. By contrast, the untreated samples showed an increase in titratable acidity after only two days ($P<0.01$), and would have been too acidic for normal processing thereafter. The total colony counts shown in *Table 2* tend to support this pattern, for the counts tended to remain stable or even decline slightly (coliform counts – $P<0.1$) in the treated samples; the higher concentrations of additives exerted little extra control of the microfloras. In the control samples, the counts increased significantly ($P<0.01$).

The reactions of the caprine milk were broadly similar, but it was noted that only the higher concentrations of reactants held the titratable acidity at or below 0.16 for 5 days at 4°C (see *Table 1*). Nevertheless, even at the lowest concentration, there was a significant delay ($P<0.01$) in the onset of acid development for two/three days. Exactly why the lactoperoxidase appears to lose its activity with time is not clear (Bjorck, 1978), but it was notable that both ovine and caprine milks had higher initial counts than the bovine samples.

The high initial total counts for the ovine milk (see *Table 2*) probably explain the high acidity of the milk, but the limited response to the thiocyanate/peroxide combination was disappointing. The more so as, according to Medina *et al.* (1989), sheep milk should contain sufficient lactoperoxidase to produce a bacteriostatic effect and the colony counts were little different from the caprine milk. Whether or not the level of catalase in the ovine milk was higher than in the caprine milk was not established, but a catalase-mediated loss of hydrogen peroxide might explain, at least in part, the contrasted results. However, given that high bacterial counts in sheep milk are not uncommon (Nunez *et al.*, 1984), activation of the LP system clearly cannot conceal poor microbiological quality (Bjorck *et al.*, 1979).

At 22°C, the untreated samples of bovine milk became unacceptable after 3 hours, and the rate of deterioration accelerated dramatically after 6 hours (see *Table 3*). However, activation of the LP system increased the shelf-life of milk samples up to 18 hours ($P<0.01$). At 30°C, the acidity of the treated bovine milks had increased little after 6 hours, whereas the acidity of the untreated samples became totally unacceptable ($P<0.05$). Indeed, even after 18 hours at 30°C, all the treated samples of cow milk would have been usable for processing, while the control had an acidity of 0.66% lactic acid. Valdez *et al.* (1988) found that the natural LP system was inhibitory against microorganisms for up to 8 hours in samples of bovine milk stored at 30°C, but it is clear

Table 2 Effect of different concentrations of SCN^- and H_2O_2 (mg/l) on the bacteriological quality of ovine, bovine and caprine milks stored at 4°C; all counts as colony-forming units/ml of milk

		Time of storage, 6 days			
Ratios	Zero	Control	15:10	75:50	100:150*
Total colony count					
(O)	1.2×10^{8}	50.0×10^{8}	1.0×10^{7}	3.5×10^{7}	8.5×10^{7}
(B)	5.3×10^{6}	10.6×10^{8}	9.4×10^{7}	6.5×10^{6}	8.2×10^{6}
(C)	4.1×10^{8}	8.5×10^{8}	3.1×10^{7}	8.5×10^{7}	2.0×10^{7}
Coliform count					
(O)	2.2×10^{5}	8.5×10^{5}	1.9×10^{3}	1.9×10^{3}	2.5×10^{3}
(B)	2.9×10^{4}	5.1×10^{6}	1.5×10^{3}	1.8×10^{3}	7.5×10^{2}
(C)	3.5×10^{6}	3.2×10^{5}	9.5×10^{2}	2.5×10^{3}	4.0×10^{3}

(O) = ovine milk; (B) = bovine milk; (C) = caprine milk.
*The results for 125:75 were similar.
Least square means: standard error = ±0.13, replications = 4.

Table 3 Effect of different concentrations of SCN^- and H_2O_2 (mg/l) on the developed acidity (% lactic acid) of ovine, bovine and caprine milks stored at ambient temperature (22°C) for up to 18 hours

Time/ratios	Control			15:10			75:50			150:100*		
(d)	(O)	(B)	(C)	(O)	(B)	(C)	(O)	(B)	(C)	(O)	(B)	(C)
0	0.18	0.14	0.12	0.18	0.12	0.12	0.18	0.13	0.12	0.18	0.14	0.12
3	0.20	0.15	0.16	0.18	0.12	0.12	0.18	0.13	0.12	0.19	0.14	0.12
6	0.27	0.24	0.22	0.18	0.13	0.13	0.19	0.14	0.13	0.21	0.15	0.13
9	0.38	0.44	0.30	0.21	0.14	0.14	0.19	0.14	0.16	0.21	0.15	0.16
12	0.45	0.53	0.38	0.20	0.14	0.14	0.20	0.15	0.17	0.25	0.15	0.18
15	0.50	0.60	0.45	0.35	0.15	0.16	0.21	0.16	0.18	0.35	0.15	0.18
18	0.60	0.62	0.52	0.38	0.16	0.25	0.38	0.17	0.30	0.38	0.16	0.26

(O) = ovine milk; (B) = bovine milk; (C) = caprine milk.
*The results for 125:75 were similar.
Least square means: standard error = ±0.07, replications = 4.

that raising the levels of thiocyanate and hydrogen peroxide can double the span of the bacteriostatic effect.

The acidity of the treated samples of caprine milk held at both room temperature and 30°C was stable for 9–12 hours even at the lowest concentrations of added reactants, while the acidity of the control was above 0.30% lactic acid. After 15 hours, the acidity of the treated caprine samples (both temperatures) was just on the borderline of acceptable, but the stability declined sharply thereafter.

Once again, it was the ovine milk that derived least benefit from activation of its LP system, with 6–9 hours being the maximum period for comparative pH stability. Obviously these values are an improvement over the controls, but it would appear that the practical benefits of adding thiocyanate / peroxide to high count ovine milks are likely to be small in the absence of refrigeration.

Overall, the results show that, by activation of the LP system in raw milk, it is possible to store ovine, bovine and caprine milks at 4°C for several days. High concentrations of sodium thiocyanate and hydrogen peroxide were only marginally more effective than the minimum levels tested, and hence a level of thiocyanate ions of 15 mg/l and H_2O_2 of 10 mg/l of milk should be sufficient to delay the risk of spoilage prior to processing; at this level, the potential health risk of the thiocyanate to humans should be negligible (Bjorck *et al.*, 1979). At higher temperatures of storage, the major advantage was observed with bovine milk, and any serious increase in acidity was inhibited for around 18 hours. With caprine and ovine milks, the high initial cell counts of the test samples tended to nullify the preservative effect of the LP system at ambient temperatures, but even so, acid development was suppressed for several hours *vis-a-vis* the control samples; 4°C and a 15:10 (mg/l) ratio of thiocyanate:hydrogen peroxide are recommended for retention of the quality in raw ovine and caprine milks. The effect of the system on the nutritional value of the milk merits further study but, even if there is an associated destruction of vitamins for example, the loss may be preferable to wasting entire batches of milk in regions where supplies are already limited.

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THE PRODUCTION OF BAFUT CHEESE — A LOCAL CHEESE FROM CAMEROON — WITH RECOMBINED AND FILLED MILKS

By

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SUMMARY

Bafut cheese is an Edam-style cheese that is popular in rural areas of the Cameroon, but shortages of fresh cow's milk limit its availability. However, cheeses made by the traditional procedure, but from filled milks (18% TS) as the raw material, proved equally acceptable to a trained panel of tasters. Locally-produced and refined palm oil was found to be a suitable source of fat (4.5% in the cheese milk), and the milk-solids-not-fat were supplied by skim-milk powder reconstituted to a level of 13.5% TS ; soya lecithin and calcium chloride were employed as aids to reconstitution and coagulation, respectively.

The finished cheese (fat 34% ; crude protein 27% ; moisture 34% ; salt 1.0%) were matured at room temperature for one month prior to consumption. It is suggested that the simple equipment and procedures involved in the production of Bafut cheese from filled milk could make the process suitable for commercial operation on a small-scale.

INTRODUCTION

The system of farming in the Cameroon is still semi-nomadic, milking is done by hand and, because the local Fulani cattle do not give high yeilds, the volume of milk available for the manufacture of dairy products is extremely limited. In recent years, the introduction of small

herds of cattle capable of providing higher yields of milk has met with some success (Herwig and Schwerdfeger, 1980) and, as a result, cheese production has started-up at a number of locations in the North-west of the country, including the village of Bafut.

The high ambient temperature, together with a lack of facilities for refrigeration, has meant that hard or semi-hard cheeses made from raw milk have become the preferred option. Many such cheeses are purely for local consumption, but one variety that is now produced in limited commercial quantities is Bafut cheese. This variety is a semi-hard, cylindrical cheese-around 2 kg in weight, with a dry rind, and typical analyses of the major components are shown in Table 1. Although the values fall broadly within the category for semi-hard cheeses (Chapman and Sharpe, 1990), variability between batches is a major problem. Given larger volumes of process milk, standardisation would become possible but, in practice, it may be many years before local agriculture can support such a demand for fresh milk.

Consequently, attempts to meet the growing consumer demand for Bafut cheese will have to rely on the use of recombined or filled milks, with the latter option being favoured by the ready availability of palm oil in Cameroon. However, a further factor that might limit development is the absence capital for large-scale plant and equipment, but this problem might be eased somewhat if filled milks with high levels of total solids could be used ; assuming that proportionately higher yields in terms of kg cheese/given volume of vat, can be obtained as the total solids are increased.

It was decided, therefore, to investigate the possibility of manufacturing Bafut cheese from recombined and filled milks and, in particular, to :

(i) prepare stable filled milks with different levels of total solids (12, 19 and 24%) ;

(ii) manufacture cheeses from these same milks to give product that were physically and chemically comparable to traditional Bafut cheese ; and

(iii) expose suitable checses to sensory analysis by a taste panel to determine whether or not the test cheeses might be acceptable in the market-place.

MATERIALS AND METHODS

The skim-milk powder was obtained from Besnier Bridel Alimentaire, France, and applying the procedures specified in Egan et al.,

1981, the important analyses of duplicate samples from a typical batch were found to be : fat < 0.5% ; protein 33.7% and lactose 51%.

Three sources of fat were considered, namely anhydrous milk fat (AMF) of local origin, crude palm oil (Trade Name — "Mulla Palm") and refined palm oil (Trade name — "Palm d'Or") both available from the Societe Camerounaise des Palmiers, Cameroon. Some important characteristics of these materials are shown in Table 2. The values for the vegetable oils are typical of the materials in question (Egan et al., 1981) and, although the figures for the local AMF were rather suspect, it was decided that all three materials would be appropriate for the manufacture of the test milks. The basic recipes for the milks were as follows :

12% TS from 9% SNF (milk powder) + 3% AMF or Oil + 0.75% lecithin.

18% TS from 13.5% SNF + 4.5 % AMF or Oil + 1.13% lecithin.

24% TS from 18% SNF + 6% AMF or Oil + 1.5% lecithin.

Food-grade soya lecithin (Fissons Laboratory Supplies, Dorset, UK) was added to all the milks at levels suggested by the manufacturer, along with 0.002 % calcium chloride as recommended by Anifantakis (1991).

Reconstitution involved heating the required quantities of fat / oil and emulsifier in a stainless steel vessel to 65°C while, at the same time, around 70% of the water was warmed to 40°C. At the desired temperature, the milk powder was incorporated into the water with a Silverson High-speed Mixer. Once the powder had dispersed, the remaining water and fat/oil were added, and the final mixture was emulsified for 15 minutes.

Control samples of local Bafut cheese were produced from fresh morning milk taken from a mixed herd of Jersey, Jersey X White Fulani and Boran X White Fulani cows mixed with an equal quantity of skimmed milk, i.e. separated milk from the previous evening ; this procedure gave milks with mean values for fat of 2.6%. Raw milk was used throughout for making the control cheeses, but the formulated milks were heat treated, after reconstitution to 12, 18 or 24% total solids (TS), at 65°C for 30 minutes.

After adjusting the temperature of the fresh or reconstituted milk to 35°C, around 4.5 ml of standard calf rennet (Chr. Hansen's Laboratory, Reading, UK)/10 litres of milk was diluted with water and poured into the vat along with required weight of calcium chloride. In the case of the recombined and filled milks, a mesophilic starter culture (CH 44) from Chr. Hansen's Laboratory was added

at the rate of 2% v/v ; the liquid starter was prepared using the procedure recommended by Tamime (1990). Stirring for 2-5 minutes ensured an even distribution of the rennet and culture, and the milk was then allowed to stand for 40-50 minutes for a firm coagulum to form.

At this point, the gel was cut with a knife into coarse cubes, and the mass left undisturbed for 15-20 minutes for the initial separation of the whey and additional acid development ; the natural microflora of the raw milk provided the source of lactic acid in the "control" cheeses, and the overnight storage of the evening milk may have encouraged the build-up of a desirable population of lactococci and/or lactobacilli (Kameni et al., 1994), The pieces of curd were then further reduced in size and allowed to settle to the bottom of the vat. After a further rest period, the surface whey was drawn-off until about 4-5 litres of curd/whey remained, and salt was added to give a level in the finished cheese of around 1.0%,

A holding period of 3-5 minutes allowed the salt to become evenly distributed, and the curd was then ladled into baskets lined with cheese cloth. The cloth was drawn around the curd to form a "ball", and manual pressure encouraged the curd to coalesce. When any given cheese no longer stuck to the cloth, the latter was removed and the cheese placed back in its basket; the final shaping of the cheese took place at this point.

The cheeses were then transferred to a well-ventilated maturation room (17-20°C). Daily turning took place until the surfaces of the cheeses were thoroughly dry, at which point the cheeses were placed on wooden shelves to complete their maturation over a period of 4-6 weeks. Regular turning and washing with saturated brine was essential to prevent the growth of adventitious moulds on the surface of the cheeses.

The final complement consisted of ten cheeses made with raw milk, and ninety test cheeses, each manufactured with either AMF, crude palm oil or refined palm oil; i.e. ten cheeses at each level of total solids for each source of fat/oil. Sub-samples from each cheese were subjected at maturity, i.e. four weeks from manufacture, to the following chemical analyses ; moisture content (Marth, 1978) ; crude protein (AOAC, 1975) ; fat (Marth, 1978) ; pH (Tamime and Robinson, 1985) ; and salt content (Egan et al., 1981). When necessary, the sub-samples of the four week-old cheeses were stored in a deep-freeze at —20°C until a specific analysis could be completed.

On the basis of the analyses for fat and moisture (see Table 3) and the desire to use a high solids milk to economise on plant, it was decided that a cheese made with a filled

or recombined milk at 18 % would be the best option, but that the choice of oil/fat should involve a taste panel analysis as well. Consequently, three samples from each group of cheeses, i.e. three raw milk products and nine test samples (3 sources of fat/oil) were subjected to sensory appraisal by a taste panel drawn from workers at the IRZV at Bamenda. The system employed was the Qualitative Descriptive Analysis approach described by Powers (1984), and the first step involved the panel deriving a series of terms to describe typical Bafut cheese. These terms were selected to cover flavour (acidity, saltiness, sweetness. and peppery), physical properties (elasticity, hardness, smoothness (absence of "grainy" mouthfeel) and close texture) and aromas (including a pleasant "aroma", i.e. cheese-like) and faulty notes (yeasty and musty). These terms were then listed on a single sheet of A 4 paper, and a 14 cm line drawn alongside each term ; vertical lines were added at points 2 cm from both ends of each line. The vertical line nearest the left-hand margin was labelled "0" indicating a low level response, whilst the opposite vertical was designated "10". At each session, seven tasters were presented with each of the four types of cheese in a random order, and were asked to score each of the selected features. Each session was repeated on three separate occasions with the order of presentation of the samples changed each time, and the overall scores for each type of cheese were analysed statistically using a standard analysis of variance procedure.

RESULTS AND DISCUSSION

The mean values for the principal components, namely fat, protein and moisture, in the control cheeses (see Table 3) were found to be not unlike many semi-hard varieties, such as Edam or Gouda (Kosikowski, 1982). However, the experimental cheeses tended to be lower in moisture than the local Bafut cheese, a pattern that no doubt reflects the increased level of fat/oil present. The mean pHs are, of course, lower than would be found with many European cheeses but, given the avenues for microbial contamination of Bafut cheese, the element of inhibition offered by the elevated acidity may not be unwelcome. The high ambient temperature of storage are, no doubt, conducive to post-production acidification, and the other biochemical reactions associated with maturation will be encouraged by the same conditions. However, the strong flavours that

Table 1 : Typical analysis of samples of Bafut cheese produced in the Cameroon ; all figures as g/100 g of cheese as consumed, and the means of twelve cheeeses

	Mean Value	Range
Fat	25.0	17.5 — 29.5
Protein	26.1	22.2 — 33.6
Moisture	41.6	40.3 — 45.1
Fat-in-Dry-Matter	42.8	38.5 — 49.5
pH	4.3	3.9 — 4.6
Salt	1.3	1.0 — 1.5

(After : Kameni et al., 1994)

Table 2 : Some chemical features of the lipid ingredients employed to manufacture Bafut cheese from recombined or filled milks.

Fat/Oil	AMF	Mulla Palm	Palm d'Or
Acid Value	0.50	0.59	0.58
Iodine Index	48.32	76.10	66.08
Peroxide value	3.02	2.69	8.05

(All figures are the means of four analyses performed according to the methods recommended by AOAC, 1975).

Table 3 : Typical analyses of samples of Bafut cheese produced from either raw milk, recombined milk or a filled milk made with either crude palm oil or refined palm oil ; all figures as g/100 g of cheese as consumed, and the means of ten cheeses for each of the levels of total solids indicated.

Fat/Oil	Fat	Protein	Moisture	pH	Salt
Raw milk	26.3	26.2	39.8	4.5	1.5
AMF					
— 12% TS	30.4	26.4	35.5	4.5	0.7
— 18% TS	33.3	26.8	32.8	4.0	0.8
— 24% TS	32.4	23.8	34.2	4.1	0.8
Crude Palm Oil					
— 12% TS	30.3	25.2	38.2	4.5	0.8
— 18% TS	34.4	24.6	34.9	4.3	0.8
— 24% TS	34.1	24.7	33.1	4.2	1.0
Refined Palm Oil					
— 12% TS	30.7	27.2	36.1	4.5	0.8
— 18% TS	34.3	26.5	33.5	4.3	0.9
— 24% TS	34.3	26.7	31.7	4.2	1.0

might be associated with lipolysis tend to be appreciated by local consumers in Cameroon, and hence it was anticipated that the taste panel might find the quality acceptable.

It was of note also that the fat levels of cheeses manufactured from reconstituted milks at 12% TS were lower than those recorded for the comparable cheeses. This problem was caused by a degree of cheesemilk instability associated with the lower total solids and, while the losses of fat during coagulation and cutting were not quantified, casual observation confirmed the presence of free fat/oil. Although the cheeses made with 18 and 24% TS were broadly comparable, the higher moisture contents of products made with the lower level suggested that 18% TS milk might be most promising for potential exploitation;

all the cheeses made for taste panel analysis were, therefore, made with 18% TS milks. The results of the taste panel analysis suggested that the members found it difficult to discriminate between the samples or, through a lack of familiarity with sensory work, were somewhat confused by the need for quantification. Nevertheless, some significant differences did emerge for certain characteristics. The prominence of a "yeasty" aroma was unexpected, because total counts for yeasts were < 200 cfu/g. However, a low level of "cheese-like" aroma and a high level of perceived "off-flavour" in cheeses produced from crude palm oil suggests that it is not a suitable source of fat for cheesemaking. Overall, the reactions to flavour were broadly comparable, and a marked "saltiness" detected in the control Bafut cheese was confirmed by the analysis (see Table 3).

The physical features of the cheeses, apart from the presence of some mechanical openings in the cheeses made with crude palm oil, suggested that no objectional characteristics would be perceived in cheeses made from the reconstituted milks. Nevertheless, the somewhat "neutral" scores for the test cheeses indicated that there is a need to improve the flavour and aroma of the cheeses made from filled milks, particularly as these oil-based cheeses will have to compete in the market with imported cheeses of European origin. If this impovement is possible, then the production of filled milk cheeses could provide a means of utilising facilities during the dry season and/or, by expanding the volume of locally-made products, reducing the current dependence on expensive, imported varieties.

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Herwig, M.K. and Schwerdtfeger

إنتاج جبن بافوت ـ (جبن نصف جاف من الكاميرون) من لبن معاد تكوينه باستخدام دهن اللبن أو الزيوت النباتية

الجبن البافوت هو صنف شبيه بالايدام ذو شعبية فى الكاميرون · إلا أن عدم توافر اللبن البقرى الطازج يحد من إنتاجه · وقد أمكن إنتاج هذا الصنف من الجبن من لبن معاد تكوينه باستخدام لبن فرز وزيوت نباتية (١٨٪ مادة صلبة) · حيث كان مقبولا للمحكمين · وقد وجد أن زيت النخيل المنتج محليا مناسبا كمصدر للدهن (٥ر٤٪ فى اللبن المعد لصناعة الجبن) بينما استخدم اللبن الفرز المجفف كمصدر لجوامد اللبن اللادهنية (٥ر١٣٪) كذلك استخدم كل من الليسيثين من الصويا وكلوريد الكالسيوم كعوامل مساعدة على استرجاع اللبن والتجبن على التوالى ·

وقد تم تسوية الجبن الناتج (٣٤٪ دهن ، ٢٧٪ بروتين خام ، ٣٤٪ رطوبه ، ١٪ ملح) على درجة حرارة الغرفه لمدة شهر قبل التسويق ·

وقد اقترح أن المعدات البسيطة والطريقة المقترحة لإنتاج الجبن البافوت من اللبن المعاد تكوينه يمكن استخدامها بكفاءة على النطاق التجارى الصغير لإنتاج هذا الصنف من الجبن ·

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Aspects of the structure of a Feta-style cheese made by direct recombination

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1. Introduction

A number of papers have confirmed that cheeses, such as Camembert and certain soft, brined types, can be made from recombined milk in which the total solids and fat/casein ratio are the same as in fresh cow milk (12, 13). However, DAVIS (4) suggested that acceptable cheeses might also be produced by directly recombining milk solids-non-fat and butterfat so as to give a cheese base with the total solids and compositional analysis to be expected in the finished cheese.

In principle, this latter approach would appear to be totally feasible, in that the proposed cheese base would not, at least in compositional terms, be unlike that obtained by ultrafiltration, and MAUBOIS and MOCQUOT (11) and others (1) have shown that cheeses can be manufactured successfully from membrane-concentrated bases.

Nevertheless, there is little information available to suggest that the structure of a cheese made the direct recombination route would be similar to its traditional counterpart. Consequently, the aim of this present study was to produce a Feta-style cheese – Gibna Baida – employing the system proposed by DAVIS (4), and to determine:

(i) whether the textural characteristics of the test cheese would be similar to normal commercial samples purchased in the Sudan; and

(ii) whether any observed differences could be correlated with contrasts in the physical structure of the cheese as revealed by electron microscopy.

2. Materials and methods

The test sample of cheese was made from a base cream composed of skim-milk powder and anhydrous milk fat blended together at 60 °C with a Silverson mixer. This cream was then ripened with a culture of *Streptococcus lactis* subsp. *lactis* and *Str. lactis* subsp. *cremoris* to give a pH of 6.5, prior to blending with sodium caseinate to give a final total solids level of 40 %, and a fat:protein ratio of 1:1. After renneting according to the procedure described by DAVIS (4), the cheese base was poured into plastic containers holding 1 kg. The individual containers were then transferred to an incubator room at 30 °C, and held at this temperature until the pH of the curd had fallen to 5.0–5.2. At this point, the solid cheeses were cooled to room temperature and removed from the containers. The individual cheeses were dry-salted (60 g/kg), and allowed to stand on draining mats overnight at 20 °C. Next morning, the cheeses were transferred to large containers of brine (10 %), and were then stored at 7 °C for one month to allow the texture of the cheese to develop.

Representative samples of:

(a) the 'cream' made from skim-milk powder and anhydrous milk fat;

(b) the cheese base, *i.e.* 'cream' plus sodium caseinate

(c) the cheese base after renneting and ripening for set periods of time; and

(d) one day-old and one month-old cheeses

were examined by both scanning and transmission electron microscopy, and the procedures are outlined in Fig. 1.

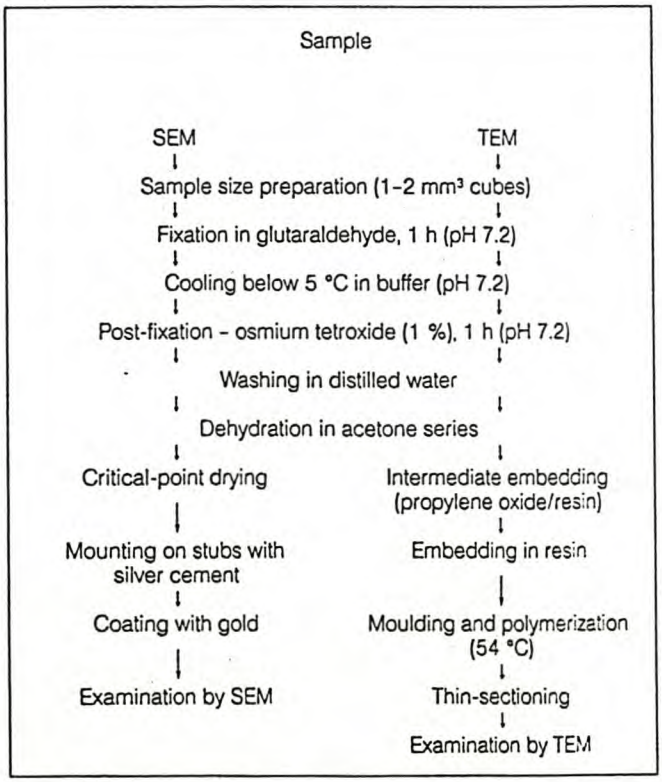

Fig. 1: General scheme for the preparation of milk and cheese samples for electron microscopy
Based upon: CARROL *et al.* (3); GREEN *et al.* (6); KALAB (8) and TARANTO *et al.* (14)

For liquid samples, an additional step involved pipetting 2 ml samples into test-tubes containing equal volumes of 25 % glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer at pH 7.2. After fixing for 1 h, the samples were mixed with an equal volume of molten agar (2 %), and poured onto a glass slide to solidify. The gel was then cut into 1–2 mm³ cubes and stored at 2 °C in 0.2 M sodium cacodylate-HCl buffer ready for further processing.

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In every case, duplicate samples of material were examined.

3. Results and discussion

Although the 'cream' was not homogenized, it was anticipated that the mixing would be sufficient to produce a stable emulsion, and Fig. 2 shows a thin-section micrograph of the emulsion prior to the addition of the sodium caseinate. It can be seen that the fat has assumed a globular form surrounded by a membrane-like surface, to which casein micelles have been adsorbed. As the lipid in the AMF would have lost most of the globule membrane material during processing, the affinity between the casein micelles and the fat globules in the aqueous phase is not unexpected. However, it is of note that the adsorption of the intact casein micelles was discontinuous, and that some of the micelles appeared to be flattened onto the surfaces of the fat globules (see Fig. 3).

Once the caseinate had been added, it formed what appeared to be a three-dimensional, background matrix providing support for the fat globules and the casein micelles. The reality of this network is suggested, in addition, by the fact that the cheese base was more viscous than the original cream. It would be expected also that this network would hinder the action of the rennet enzyme, and this conclusion was supported by the observed results.

Thus, the thin sections of cheese base shown in Fig. 4 indicate that the enzyme has had no noticeable effect on the casein micelles and/or caseinate matrix even 5 min after addition. This lack of activity is in marked contrast to the behaviour of rennet in fresh milk (2, 5).

Whether the extreme buffering capacity of the system, and resultant high pH of the base, played a part is not clear, but the work of GREEN (7) implies that a reduction in the mean free path for the micelles, in comparison with normal milk, may be more important.

Ten min after the addition of the rennet, a few micelles were observed to form a chain structure of some 2/4 units, but it was not until 1 h later that electron-transparent 'gaps' could be seen in the protein matrix, indicating, in all probability, shrinkage of the caseinates towards the casein micelles and/or further coalescence of the micelles themselves.

However, after 1 day, the young cheese had developed a definite texture, and as shown in Fig. 5, the micelles and caseinate had coalesced into large, rough-edged aggregates which trapped fat globules between them; some interspaces, probably whey-filled, were also evident.

This structure was confirmed by the scanning electron micrograph (Fig. 6) of a one day-old cheese, which shows quite long, continuous masses of intimately related fat and protein; after 1 month, the picture was broadly similar, except that the protein chains had tended to become somewhat shorter. The structure of the 'Gibna Baida' was, however, entirely different, in that the curd exhibited a directional pattern of fusion with clearly ruptured fat globules (see Fig. 7). Whether this contrast is a reflection of the fact that 'Gibna Baida' is a structured cheese, *i.e.* subject to pressing, whereas the test cheese is unstructured is a matter for speculation, but it is of note that the overall structure of the experimental cheese closely resembled the cream cheese described by KALAB *et al.* (10) in which the fat globules were cemented together by the milk proteins.

For some practical applications, these differences in the fine structure of the finished cheeses did not prove to be important. In particular, both the test cheese and the Sudanese cheese showed the same tendency to fracture in a clean, conchoidal manner, and both could be crumbled manually with equal facility. The sprinkling of either cheese over a salad would, therefore, cause no problems, but the melting properties of the two cheeses were markedly different. In particular, the test cheese tended to lose coherence, and the contrast in structures (see Fig. 6, 7) would tend to support this pattern of behaviour.

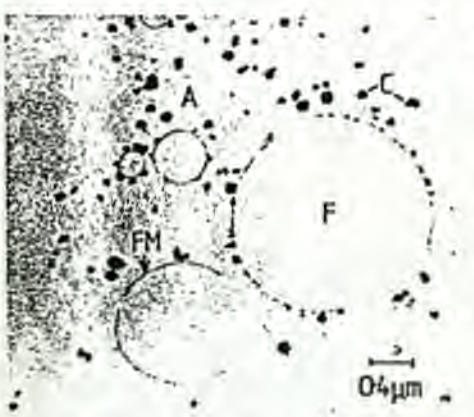

Fig. 2: Transmission electron micrograph of a thin-section of the 'cream' made from skim-milk powder and anhydrous milk fat. The casein micelles (C) can be seen dispersed in the aqueous phase (A), or adsorbed to the membrane-like surface (FM) of the fat globules (F).

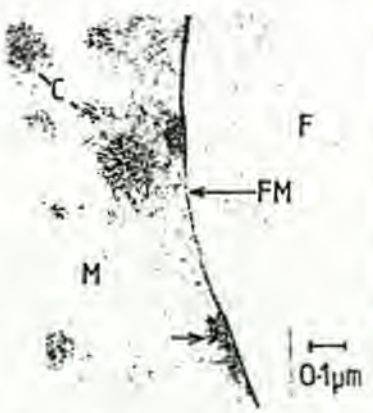

Fig. 3: Transmission electron micrograph of a thin-section of the 'cheese base' made from sodium caseinate, skim-milk powder and anhydrous milk fat. The casein micelles (C) can be seen flattened over the membrane-like surface (FM) of the fat globules (F); the background matrix (M) formed by the caseinate is also in evidence.

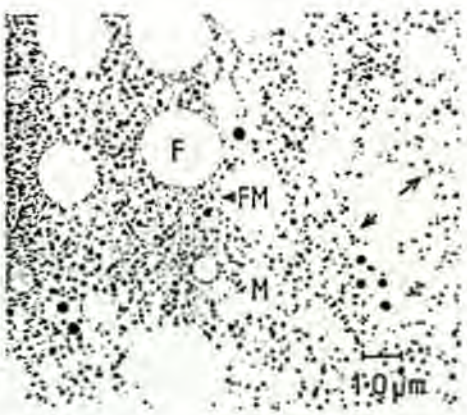

Fig. 4: Transmission electron micrograph of a thin-section of the 'cheese base' five min after the addition of rennet. The membrane-like surfaces (FM) of the fat globules (F) appear somewhat 'sharper', but the background matrix (M) seems to have been unaffected by the enzyme.

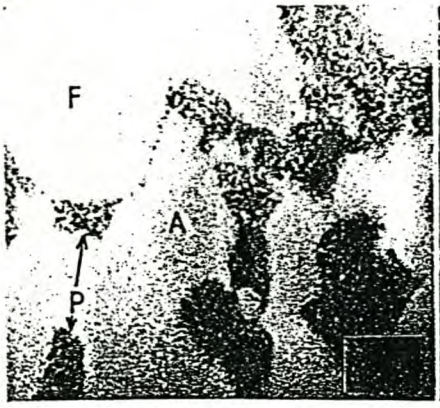

Fig. 5: Transmission electron micrograph of a thin-section of a one day-old cheese. Chains of coalesced protein (P) are now evident joining the fat globules (F), and the matrix of caseinate appears to be contracting to give denser regions (A) and 'gaps' which, at this stage, probably contain whey.

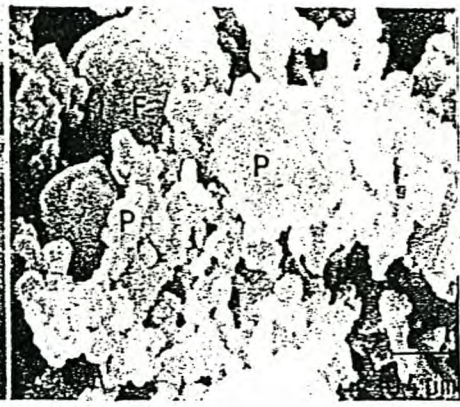

Fig. 6: Scanning electron micrograph of a one-day old cheese. Chains of coalesced protein (P) are evident around the fat globules (F), together with whey-filled spaces (A).

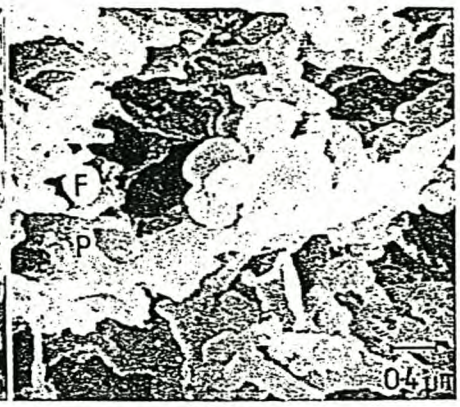

Fig. 7: Scanning electron micrograph of a market sample of Sudanese 'Gibna Baida'. Although chains of coalesced protein (P) are evident, as are fat globules (F) and whey-filled spaces (A), the nature of the protein fusion appears quite different.

Nevertheless, despite some limitations in respect of functional properties, the manufacture of a Feta-style cheese by direct recombination is clearly a feasible proposition, and from readily available raw materials. The advent of high-protein skim-milk powders offers yet another alternative, and certainly some regions of the world will find this technology most attractive.

Acknowledgements

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5. Summary

ALI, M.Z., ROBINSON, R.K.: Aspects of the structure of a Feta-style cheese made by direct recombination. Milchwissenschaft 45 (11) 699-701 (1990).

51 Feta cheese (recombination)

A Feta-style cheese was produced by the direct combination of anhydrous milk fat, sodium caseinate and skim-milk powder. The textural and organoleptic properties of the end-product were similar to a popular Sudanese cheese 'Gibna Baida', but the fine structure, as revealed by the electron microscope, was markedly different. Contrasts in the fat/protein relationship were especially notable.

ALI, M.Z., ROBINSON, R.K.: Aspekte der Struktur eines Feta-ähnlichen Käses. Milchwissenschaft 45 (11) 699-701 (1990).

51 Feta-Käse (Rekombination)

Ein Feta-ähnlicher Käse wurde durch direkte Kombination von wasserfreiem Milchfett, Natriumcaseinat und Magermilchpulver hergestellt. Textur und organoleptische Eigenschaften des Endprodukts ähnelten denen eines verbreiteten sudanesischen Käses „Gibna Baida". Allerdings war die Feinstruktur, wie sich unter dem Elektronenmikroskop zeigte, deutlich abweichend. Es waren insbesondere Unterschiede im Fett/Protein-Verhältnis festzustellen.

ALI, M.Z., ROBINSON, R.K.: Aspects de la structure d'un fromage du type Feta produit par recombination directe. Milchwissenschaft 45 (11) 699-701 (1990).

51 Fromage Feta (recombination)

ALI, M.Z., ROBINSON, R.K.: Aspectos de la estructura de un queso del tipo Feta manufacturado por recombinación directa. Milchwissenschaft 45 (11) 699-701 (1990).

51 Queso Feta (recombinación)

66 عـدد ٦٣ ١٩٨٢

SUDAN NOTES AND RECORDS

incorporating
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Philosophical Society
of the Sudan

المدرجة فيها وقائع الجمعية الفلسفية بالسودان

VOLUME LXIII 1982

A SURVEY OF CHEESEMAKING IN THE AL-DUWAYM AREA

by

MOHAMMED ZEIN ALI and R K ROBINSON

The cheese traditionally produced in the Sudan is a white-brined variety known locally as *jibna bayḍā'* ('white cheese'), and is widely consumed by people of all socio-economic classes. It is used in different ways, for example, as a dressing for salads and *fūl miṣrī* (a kind of bean) and in sandwiches. Most of it is made in farmhouses, while small quantities are manufactured by government institutions such as animal resources departments and the universities.

1 Historical Background

Although the lack of historical records makes it difficult to date exactly the evolution of the process, Abdulla (1975) mentions that cheesemaking around al-Duwaym (200 kilometres south of Khartoum on the west bank of the White Nile) was, during the period of the Turko-Egyptian regime, a rather simple process. Once collected, the milk was placed in containers, and the crushed fruits of a wild plant known locally as *jubayn* (*Solanum dubium*) were added to curdle it; the whey was then filtered off, leaving a solid mass. The process remained largely unchanged until a Greek couple, Katharina and Panaioti Maestro, settled at al-Duwaym in 1908 and pioneered the production of *feta*-type cheese. Initially, cheesemaking was a small-scale, backyard industry, and the product was distributed to relatives and friends and then to high-ranking British and Egyptian officials. Stimulated by the growing demand, the Maestro family opened their first factory in 1920 (El Huda 1964; El Sayed 1971).

A second factory was established in 1932 by another Greek merchant, Philip Kadaras (Abdulla 1975). The industry grew quickly, and more investors, including Sudanese and Greek merchants, joined in, tempted by the low price of milk collected from the nearby villages and the high profits. However, the expansion of the industry was not accompanied by any concern on the part of the local authorities about public health measures or quality control, though land charges and taxes were levied, and licensing regulations had become, officially, more strict by the 1960's (Trilsbach 1978). Riyāḍ (1977) estimated the amount of cheese produced in the Sudan during 1975/76 at 8 million kg, and that of clarified butter at 5 million kg.

Jibna bayḍā' is a crumbly white variety of cheese similar to the Egyptian *dimyāṭī* and Greek *feta* as far as flavour, salty taste and texture are

concerned. There are some other varieties of cheese produced in the Sudan, though in negligible quantities, such as cream cheese and *maḍāfara*; the latter is of Syrian origin and is textured by stretching the heated coagulum in 'flakes'.

Cheese consumption has been increasingly encouraged by both governmental and private agencies over the last three decades, and cheese has become one of the most important items of food for breakfast and supper served by the catering departments of schools and universities. Trilsbach (1978) estimated the cost of production of 10 kg of *jibna bayḍā'* at Ls2.10 in 1971 and Ls12.77 in 1980, while the retail price of one kilogram increased from about Ls0.60 in 1971 to Ls8–9 by early 1984.

It is expected that cheese consumption will increase significantly over the next few decades, and this trend, coupled with shortages and rising retail prices, will put considerable pressure on the dairy industry to increase supplies of local cheeses.

2 The Survey

The north-western part of the Jazīra Region, formerly the northern part of White Nile Province, is the area best known for cheese production in the Sudan. Lying within the geographical boundaries of the semi-desert, low-woodland savannah zone, it is characterized by a seasonal rainfall between July and October and is traversed by the White Nile. It is an important livestock-rearing area, with cows (Zebu-type), sheep and goats for milk production under nomadic and semi-nomadic land-use patterns. Vast quantities of milk from these different species are produced in the region, and owing to the limited local consumption the surplus goes into cheesemaking. Most of the farmhouses are in the vicinity of the town of al-Duwaym and are usually named after the location of the family which owns them. Officially, the North-west White Nile District Council, the local authority of the cheesemaking area, recognizes 140 farmhouses where cheese is made, the greatest concentration being to the south and west of the town.

In order to survey the method of manufacture of *jibna bayḍā'* in the Sudan, one of the authors (M Z A) travelled to the region early in 1983 and, taking al-Duwaym as a centre, visited six small cheesemaking farmhouses within a radius of 45 km.

The basic method of cheesemaking is outlined below, together with the results of some physico-chemical analyses of typical cheese samples (see Fig. 1 and Table I).

3 The Farmhouse Process

The milk, usually a mixture of cows', sheep's and goats' milk, is collected in the morning, starting at about 6 o'clock, from the sellers, who

bring their produce to the cheesemaking premises, and stored in galvanized steel drums of 200–220 litres' capacity. Collection may extend over 5–6 hours, and cheesemaking commences when a full drum of milk has accumulated. The amount of milk collected by a farmhouse varies in accordance with the annual fluctuation of rainfall; the range is between two and five drumfuls a day in the dry and wet seasons respectively. After collection, the milk is poured into another clean drum through cheesecloth stretched across the top, and salt (10–15 kg, 5–10%) is added by placing it on top of the cloth. Although the raw milk is not pasteurized, it is common practice to warm it to 30–35°C during the winter season. The quantity of salt can vary considerably, and may be up to 20% (w/v) during the summer.

Four or five standard rennet tablets (8 or 10 g)(Chr. Hansens Laboratorium A/S, Denmark, or Glad and Co. A/S, Denmark) are crushed, dissolved in a small amount of water and then added to a drum of salted milk; slightly higher levels (10–12 g) may be used in winter to achieve better coagulation. The mixture is stirred manually with a long wooden paddle for one or two minutes, and the drum is covered with clean cheesecloth and left undisturbed for 4–6 hours to coagulate.

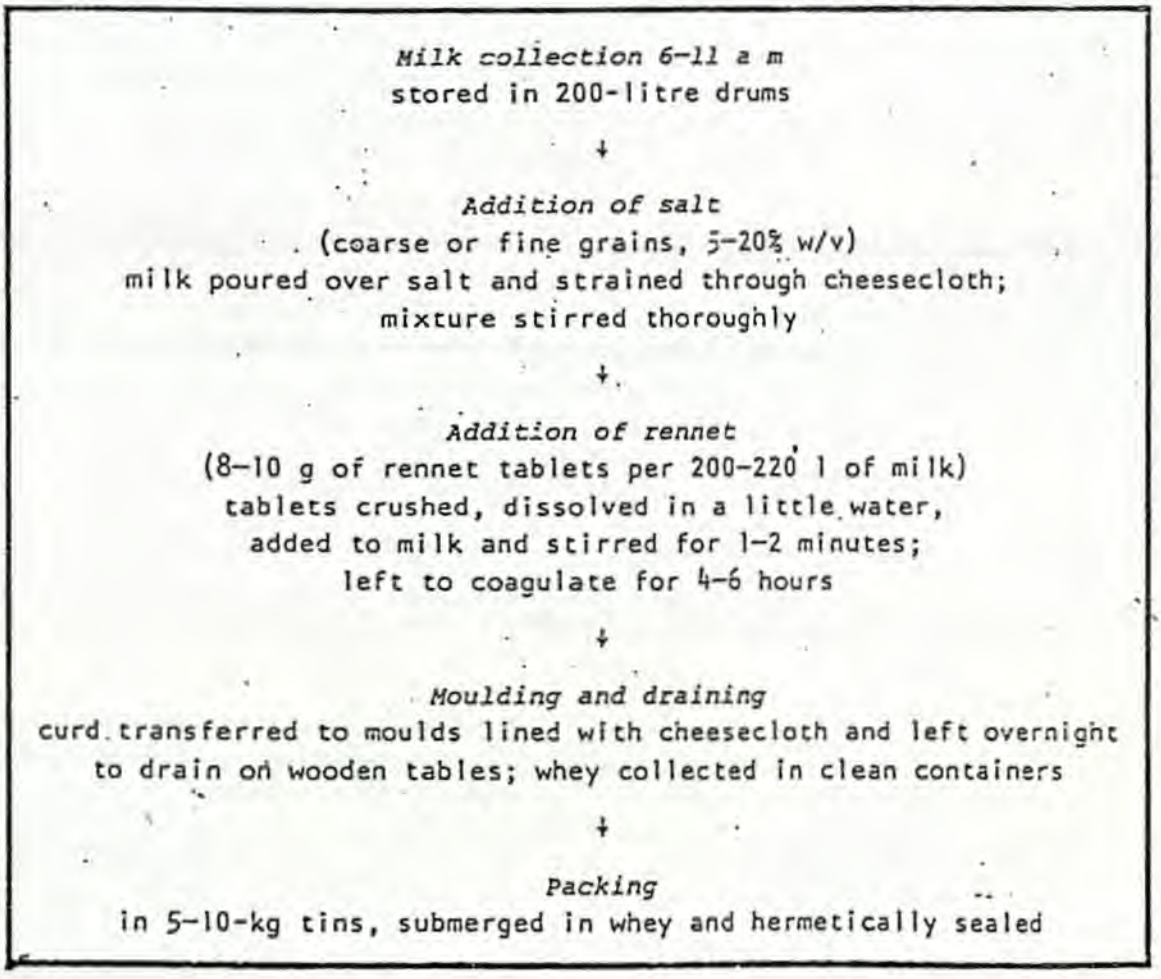

Fig. 1: Basic Schedule for the Production of Jibna Bayḍā' *in the Farmhouse*

The firm curd, as assessed by visual and tactile observation, is gently broken, transferred to moulds lined with cheesecloth and left to drain on wooden tables for about fifteen hours, and the whey is collected in clean metal containers. The young cheese is placed in tins of 5–10-kg capacity, which are left unsoldered overnight to allow additional drainage. The cheese is then covered with the collected whey, and the tins hermetically sealed. These are transported to al-Duwaym, where the farmhouse labels are affixed, and either transported directly to areas of demand, such as the Three Towns, or stored for a few days before marketing.

4 Results of Physico-chemical Analyses of Jibna Bayḍā'

Cheese samples were obtained from the retail shop of each of the six farmhouses and analyses carried out to determine the moisture content, titratable acidity (TA), pH, and percentages of protein, fat and salt. The results are shown in Table I. The moisture content ranged between 55 and 59%, and according to Scott (1981) Sudanese *jibna bayḍā'* could therefore be classified as a soft cheese. The salt content, one of the important characteristics of this cheese, showed some variation between samples obtained from different farmhouses (6–9%); it is similar to that of Egyptian *dimyāṭī* and Greek *feta*.

Table I: Results of Analyses of Samples of Jibna Bayḍā'

No.	*Parameter*	*Reference Method*	*Commercial Cheese Samples*[A] 1	2	3	4	5	6	*Mean*	*Stand. Deviation*
1	Moisture content	Brit. Stand. 1976	56.21	58.91	55.62	56.80	54.32	57.91	56.63	1.49
2	TA: per cent lactic acid	Egan *et al.* 1981	0.98	0.86	0.94	1.20	0.92	0.95	0.975	0.107
3	pH	Brit. Stand. 1976	4.9	5.1	4.8	4.7	5.0	4.9	4.9	0.13
4	Crude protein (T N × 6.38)	Egan *et al.* 1981	14.1	11.2	16.2	15.1	14.3	11.7	13.77	1.78
5	Fat content (%)	Brit. Stand. 1969	14.5	12.0	13.5	16.0	15.5	12.5	14.0	1.47
6	Salt content (%)	Brit. Stand. 1976	6.8	6.9	8.6	8.5	9.1	7.7	7.9	0.87

[A]*Each reading is an average of those obtained from duplicate samples*

5 Comments on Farmhouse Cheesemaking

It is clear that most cheesemaking in the Sudan is basically a cottage industry, the equipment is simple and can be made locally, and the procedure is suited to small-scale production. It is worth mentioning that most of the farmhouse owners have more than two plants in different locations, so that staff and equipment can be moved from one site to another according to the

availability of milk; that is, they can follow the nomads as they search for pasture and water. Although this flexibility in production is essential, the authors believe that the standards of hygiene in the farmhouses visited were very poor, and that the quality of the cheese was adversely affected. The farmhouses are by their nature very difficult to control, and the following observations reflect this problem:

i The location of these plants in remote pastoral areas has made it very difficult for them to have access to such basic industrial assets as water, electricity and paved roads. Also, the seasonality of milk production dictates that these premises may be unmanned for much of the year.

ii There is no scientific check on the quality of the milk, and the staff, usually 1–4 casual labourers, are mostly unaware of the basic essentials of hygiene: for example, their bedding can often be seen in the cheese store or the processing room.

iii The local authorities, in particular health officers, cannot cope with the inspection of such a large geographical area as that which is within their jurisdiction.

iv The problem of animal feed shortages under the existing nomadic pattern of livestock rearing could well affect the quantity and the quality of the product, as could improper and inadequate medication of the livestock.

v Finally, the production process is not standardized and can be influenced by many factors, such as location, season, consumer preference, intended storage period and quality of the milk.

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ORIGINAL ARTICLE

Selection of starter cultures for the fermentation of soya milk

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The growth of four cultures selected on the basis of their ability to produce lactic acid in soya milk and/or utilize oligosaccharides, namely Streptococcus thermophilus, Lactobacillus delbrueckii *subsp* bulgaricus, Lactobacillus fermenti *and* Lactobacillus fermentum, *was examined in reconstituted, low fat, spray-dried soya milk powder (12% total solids). The single culture of* S. thermophilus *produced a drop in pH from 6·5 to 4·7 over a 10-h period, and reduced the level of stachyose from 8·5 mg ml^{-1} in the original milk to 3·2 mg ml^{-1}; after 24 h fermentation, pH fell to 4·5, and the stachyose concentration to 3·0 mg ml^{-1}. The paired culture of* S. thermophilus *and* L. fermentum *behaved in a similar fashion, but with only a slight improvement in stachyose utilization. When yeast extract (0·3%) and glucose (1·0%) were added to the soya milk, acid production by all the cultures increased dramatically, and* L. delbrueckii *subsp* bulgaricus *alone or in combination with* S. thermophilus *lowered the pH of the milk to 4·3 over 10 h. This combination of* S. thermophilus *and* L. delbrueckii *subsp* bulgaricus *was considered to be a likely combination for the production of a fermented product from soya milk or modified soya milk, as neither* L. fermenti *nor* L. fermentum *were appreciably more effective in lowering the concentrations of oligosaccharides.*

Introduction

The soya bean, *Glycine max.* L. Merr., a member of the family Leguminosae, originated in the Far East, and has provided food for that part of the world for thousands of years. Thus, although soya beans are one of the most important oilseeds in the world (Smith and Huyser 1987), they are also high in protein, and a typical analysis (dry weight basis) might be 20% lipid, 40% protein, 35% carbohydrate and 5% ash (Pearson 1983). However, despite this attractive composition, whole soya beans are consumed only infrequently, and most Asian populations use them as a base for the production of fermented foods (Fukushima 1981). In such foods, the soya protein is more than 90% digestible—approximately the same as meat, egg and milk protein and, in recent years, these traditional soya foods have attracted interest among various populations in Western countries (Messina and Messina 1993).

Some soya dishes use tofu (soya bean curd) as a base, while products like soya sauce and miso are popular as flavourings (Cook 1993). Various soya cheeses are made in countries

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like Taiwan, Vietnam, The Philippines, Thailand, Hong Kong and Korea (Hesseltine 1993) but, as many rely on the use of heat and salts/lactic acid (magnesium sulphate, calcium sulphate or glucono-δ-lactone) to produce the gel, the texture of the final products is often unsatisfactory (Kohyama and Nishinari 1993). However, if cheese-like products could be made using soya milk and lactic acid bacteria, then the slower coagulation might give rise to a product with a more consistent texture.

Many attempts to follow this route have failed because: (1) soya milk has sucrose as the main fermentable sugar and only limited amounts of glucose; (2) even if the lactic acid bacteria have grown, a vile tasting product is formed (Hesseltine 1993). Furthermore, the oligosaccharide constituents of soya milk, such as raffinose and stachyose, are not hydrolysed by most lactic acid bacteria, and hence remain a problem with respect to their flatulence-inducing properties (Rackis et al. 1967); some problems with a residual 'beany' flavour have been cited as an additional barrier to exploitation. Nevertheless, a number of factors would seem to suggest that a soya cheese would be popular with many consumers: (1) cheese made from mammalian milk is a major food item around the world, and has an excellent 'image'; (2) countries like Thailand or Indonesia have an abundance of soya beans, but little surplus cow or other milk; (3) in Thailand in particular, imported cheeses like Cheddar are too expensive for many consumers.

It is also important that many countries in the Far East grow and consume many types of legume in addition to soya, so that a technology applicable to soya might be considered for the exploitation of other beans as well. However, if a legume-based cheese is to become a reality, the first essential is the selection of a bacterial culture capable of completing the coagulation stage; hence the aims of this project were to:

(1) examine the performance in soya milk of a range of lactic acid bacteria that might be used as starter cultures in a cheese-making process; only thermophilic cultures were considered as: (a) fermentation at 37–40°C should achieve more rapid acidification—optimum gel formation of soya milk occurs at pH 4·5, and (b) thermophiles have been used successfully to make soya yoghurt (Tamime and Robinson 1985);
(2) determine whether or not the same cultures would utilize the flatulence-inducing oligosaccharides during fermentation.

Materials and Methods

Preparation of soya milks

Low fat, spray-dried soya milk powder supplied from Allergycare, Pollards Yard, Taunton, UK was used as the base material, and it was dissolved at the rate of 120 g^{-1} in water deionized by reverse osmosis. After mixing with a high-speed emulsifier/mixer (Silverson Machines Ltd., Waterside, Chesham, UK), the suspension was transferred to 250 ml screw-cap bottles (100 ml amounts) and sterilized by autoclaving at 121°C for 15 min; the milks were then cooled and stored at 4°C before use.

As a more nutrient-rich alternative, yeast glucose soya milk (YGSM) (Pinthong et al. 1980a) was prepared by adding 3 g yeast extract (Code L21, Oxoid, Basingstoke, Hants, UK) and 10 g D-glucose to 1 l of soya milk; the medium was handled as described for the standard soya milk.

Starter cultures

The standard cultures employed for yoghurt making, namely *Streptococcus thermophilus* NCDO 1496 and *Lactobacillus delbrueckii* subsp *bulgaricus* NCDO 1489, were obtained from the Dairy Microbiology Laboratory, Department of Food Science and Technology, Reading University, Reading, UK. However, although these isolates were known to be capable of growth in soya milk, it was not known if they could ferment oligosaccharides. Consequently, cultures of *L. fermenti*

NRRC 207 from the Northern Regional Research Laboratory, Peoria, USA and *L. fermentum* NCDO 1750 (National Collection Food Bacteria, Aberdeen, UK) were selected as complementary species that might degrade raffinose or stachyose.

Propagation of the starter cultures

Stock cultures of the bacteria were grown in the following media: *S. thermophilis* in M17 broth, *L. delbrueckii* subsp *bulgaricus* in skimmed milk, and *L. fermenti* and *L. fermentum* in MRS broth; all cultures were incubated at 37°C for 24 h and then kept at 4°C until further use. The subcultures employed for the experimental work were prepared weekly by inoculating 1% (v/v) of an existing culture into 100 ml of sterilized soya milk and incubating at 37°C for 12–16 h.

Physico-chemical analysis of soya milk powder

The moisture and total solids of a sample of powder were determined according to ADAS (1986), as was the ash content. The fat content was measured by extraction with petroleum ether in a Soxtec extractor (ADAS 1986), while the concentration of starch was determined by enzyme hydrolysis (Macrae and Armstrong 1968). The water-soluble carbohydrates were extracted with water and, after filtration, the extract was treated with 0·133 M sulphuric acid before analysis using a continuous flow autoanalyser (Smith et al. 1964). The total nitrogen content of the sample was found using a Leco Nitrogen Determinator (Leco Instruments Ltd., Stockport, UK), and the protein content was calculated as total nitrogen multiplied by 6·25.

Determination of growth and acid production in soya milk and yeast glucose soya milk

Bottles of sterile soya milk (100 ml, 12% TS) or YGSM were inoculated (1% v/v) from 24 h-old cultures of the following single species: *S. thermophilus*, *L. delbrueckii* subsp *bulgaricus*, *L. fermenti* and *L. fermentum*. Mixed cultures (1:1 ratios, 1% v/v) of *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus*, *S. thermophilus* and *L. fermenti* and *S. thermophilus* and *L. fermentum* were also tested. The inoculated media were incubated at 37°C and, at the time of inoculation (0 h) and at 2-h intervals thereafter, duplicate bottles of each culture/pair of cultures were withdrawn from the incubator. A sample (1 ml) of culture was aseptically withdrawn from each bottle in order to record the total colony counts, and additional samples (10 ml) were extracted for measurement of pH.

Serials dilutions of the samples (1 ml) were made in maximum recovery diluent (MRD) (Code: CM733) supplied by Oxoid Ltd., Basingstoke, Hants, UK and 0·1 ml amounts from dilutions 10^{-2}–10^{-7} were spread onto the surfaces of predried plates of M17 agar for *S. thermophilus* and acidified MRS agar (pH 5·4) for *L. delbrueckii* subsp *bulgaricus*, *L. fermenti* and *L. fermentum*. Duplicates plate were incubated for up to 48 h at 37°C.

The pH values of the samples were determined with a pH meter (Kent EIL 7020) provided with a combined electrode (BDH, Gelplas, UK); the machine was calibrated before use with buffer solutions of pH 7·0 and 4·0.

Utilization of oligosaccharides in soya milk

The same cultures were inoculated (2% v/v in order to provide a high cell as soon as possible) into 100 ml amounts of soya milk (12% TS) in 250 ml bottles, and the inoculated milks were incubated at 37°C. At the time of inoculation (0 h), and after 10 and 24 h samples (10 g) were removed for analysis of sucrose, stachyose and raffinose.

The extraction method was based on those described by Pinthong et al. (1980b) and Knudsen (1986). The overall procedure is outlined in Fig. 1, and it differs from the published methods in that the extraction with chloroform was completed first; Pinthong et al. (1980b) carried out the initial extraction with ethanol, and then removed residual fat from the ethanol extract. The final solution

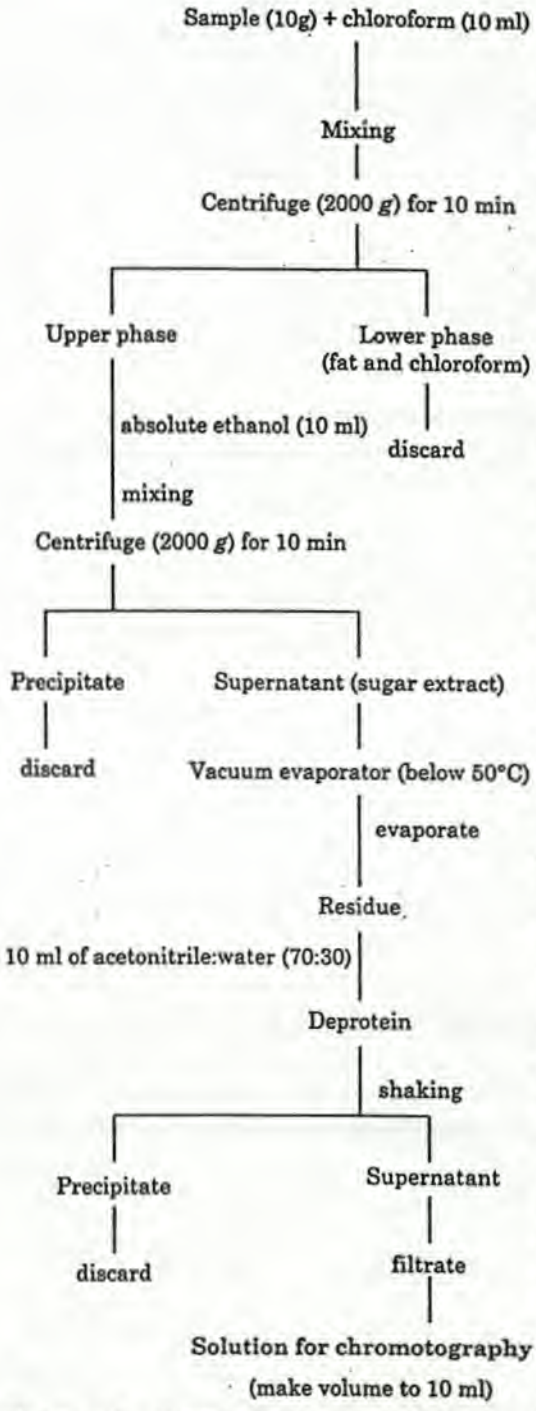

Figure 1. Procedure for the extraction of oligosaccharides.

was used directly for high pressure liquid chromatography (HPLC), the observed peaks were compared with the peak height of standards of known concentration. The chromatography system included an evaporative-light-scattering detector, and a Techsphere-NH_2 micro-column (150 × 2 mm internal diameter) from HPLC Technology Company, Macclesfield, UK. Sample injection was carried out by means of a fixed loop (10 µl). The mobile phase was acetonitrile-water (70:30; v/v) with a flow rate of 0·2 ml min^{-1}.

Results and Discussion

The chemical composition of the soya milk powder was crude protein 49·3%, water-soluble carbohydrates 9·4%, starch 1·0%, fat 9·2%, ash 5·1% and moisture 3·1%, so that the composition of the milk (12%, w/v) was 5·9 protein and 1·1% fat. It was confirmed that the major sugars in the soya milk powder were sucrose (5%), raffinose (1·1%) and stachyose (3·8%). Since sucrose is a readily fermentable sugar, appropriate species of bacteria should be able to produce substantial amounts of lactic acid in soya milk (Angeles and Marth 1971), but supplementation with other carbohydrates (glucose) and/or growth stimulants (yeast extract) may be necessary to ensure adequate acid production by some cultures.

S. thermophilus grew rapidly in soya milk, and achieved a maximum population of $1·74 \times 10^{10}$ cfu ml^{-1} after 10 h, while in YGSM a count of $1·01 \times 10^{10}$ cfu ml^{-1} was only reached after 24 h in YGSM, however, there was a stimulation of acid production, and the pH of the milk reached 4·2 in 24 h compared with 4·5 in soya milk. The growth of *L. delbrueckii* subsp *bulgaricus* was similar in both YGSM ($2·85 \times 10^{10}$ cfu ml^{-1}) and soya milk ($4·00 \times 10^{10}$ cfu ml^{-1}) over the first 10 h, but then the populations stabilized (see Tables 1 and 2). There was, however, a contrast with respect to pH; a value of 4·3 was reached in 10 h in YSGM but, in soya milk, the pH was only 5·1 after 24 h. Whether or not the glucose acted as a stimulant for acid production was not established, but the restricted ability of *L. delbrueckii* subsp

bulgaricus to utilize the oligosaccharides (see Table 3) may be relevant.

The growth of *L. fermenti* in YGSM was much better than in soya milk, even though utilization of oligosaccharides might have been expected to reduce the contrast (Pinthong et al. 1980b); however, the inability of this isolate to metabolize sucrose could have retarded its growth in soya milk. Thus, the maximum count in YGSM was $1{\cdot}48 \times 10^{10}$ cfu ml^{-1} after 10 h, but only $2{\cdot}83 \times 10^{8}$ cfu ml^{-1} in soya milk; this contrast was reflected in pH values recorded, for while *L. fermenti* reduced the pH in YGSM to 4·5 in approximately 10 h, the pH of soya milk had only dropped to 5·9.

Even though *L. fermentum* should be able to utilize oligosaccharides and grow more rapidly in soya milk, the final pH values were 4·3 in YGSM and 4·9 in soya milk after 24 h; this pattern is close to that of *L. delbrueckii* subsp *bulgaricus*.

The final counts of *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* (1:1) in the

Table 1. pH of the milk and numbers of colony forming units (cfu) of lactic acid bacteria growing at 37°C for 10 and 24 h in soya milk (12% TS)

	Soya milk (12% TS)					
	Initial range		After 10 h		After 24 h	
Micro-organisms	pH	cfu ml^{-1}	pH	cfu ml^{-1}	pH	cfu ml^{-1}
S. thermophilus	6·5	$1{\cdot}01 \times 10^{7} \pm 0{\cdot}45$	4·7	$1{\cdot}74 \times 10^{10} \pm 0{\cdot}05$	4·5	$3{\cdot}20 \times 10^{9} \pm 0{\cdot}02$
L. delbrueckii subsp *bulgaricus*	6·5	$2{\cdot}41 \times 10^{7} \pm 0{\cdot}06$	5·5	$4{\cdot}00 \times 10^{10} \pm 0{\cdot}08$	5·1	$3{\cdot}23 \times 10^{10} \pm 0{\cdot}08$
L. fermenti	6·4	$2{\cdot}24 \times 10^{7} \pm 0{\cdot}27$	5·9	$2{\cdot}83 \times 10^{8} \pm 0{\cdot}09$	4·9	$1{\cdot}24 \times 10^{8} \pm 0{\cdot}04$
L. fermentum	6·4	$7{\cdot}93 \times 10^{7} \pm 0{\cdot}47$	5·0	$9{\cdot}35 \times 10^{9} \pm 0{\cdot}29$	4·9	$5{\cdot}88 \times 10^{9} \pm 0{\cdot}22$
S. thermophilus + *L. delbrueckii* subsp *bulgaricus*	6·5	$6{\cdot}45 \times 10^{6} \pm 0{\cdot}35$ $1{\cdot}77 \times 10^{6} \pm 0{\cdot}06$	4·8	$2{\cdot}08 \times 10^{10} \pm 0{\cdot}15$ $2{\cdot}59 \times 10^{10} \pm 0{\cdot}19$	4·5	$5{\cdot}54 \times 10^{10} \pm 0{\cdot}12$ $7{\cdot}18 \times 10^{10} \pm 0{\cdot}20$
S. thermophilus + *L. fermenti*	6·5	$2{\cdot}10 \times 10^{6} \pm 0{\cdot}09$ $6{\cdot}35 \times 10^{5} \pm 0{\cdot}01$	5·1	$1{\cdot}04 \times 10^{9} \pm 0{\cdot}45$ $8{\cdot}57 \times 10^{8} \pm 0{\cdot}05$	4·4	$3{\cdot}20 \times 10^{8} \pm 0{\cdot}43$ $5{\cdot}65 \times 10^{8} \pm 0{\cdot}38$
S. thermophilus + *L. fermentum*	6·5	$1{\cdot}18 \times 10^{7} \pm 0{\cdot}20$ $7{\cdot}05 \times 10^{6} \pm 0{\cdot}16$	4·7	$9{\cdot}00 \times 10^{9} \pm 0{\cdot}25$ $4{\cdot}48 \times 10^{9} \pm 0{\cdot}47$	4·6	$7{\cdot}00 \times 10^{9} \pm 0{\cdot}01$ $6{\cdot}95 \times 10^{9} \pm 0{\cdot}26$

All results are the means of two identical experiments.

Table 2. pH of the milk and numbers of colony-forming units (cfu) of lactic acid bacteria growing at 37°C for 10 and 24 hours in yeast glucose soya milk

	Yeast glucose soya milk					
	Initial range		After 10 h		After 24 h	
Micro-organisms	pH	cfu ml^{-1}	pH	cfu ml^{-1}	pH	cfu ml^{-1}
S. thermophilus	6·3	$1{\cdot}45 \times 10^{7} \pm 0{\cdot}30$	4·6	$2{\cdot}70 \times 10^{9} \pm 0{\cdot}17$	4·2	$1{\cdot}01 \times 10^{10} \pm 0{\cdot}36$
L. delbrueckii subsp *bulgaricus*	6·4	$1{\cdot}83 \times 10^{6} \pm 0{\cdot}10$	4·3	$2{\cdot}85 \times 10^{10} \pm 0{\cdot}22$	4·1	$4{\cdot}39 \times 10^{10} \pm 0{\cdot}02$
L. fermenti	6·3	$2{\cdot}08 \times 10^{7} \pm 0{\cdot}37$	4·5	$1{\cdot}48 \times 10^{10} \pm 0{\cdot}24$	4·5	$6{\cdot}71 \times 10^{9} \pm 0{\cdot}11$
L. fermentum	6·4	$4{\cdot}00 \times 10^{7} \pm 0{\cdot}24$	4·4	$1{\cdot}20 \times 10^{10} \pm 0{\cdot}07$	4·3	$2{\cdot}09 \times 10^{9} \pm 0{\cdot}06$
S. thermophilus + *L. delbrueckii* subsp *bulgaricus*	6·2	$2{\cdot}69 \times 10^{6} \pm 0{\cdot}37$ $7{\cdot}60 \times 10^{5} \pm 0{\cdot}02$	4·3	$1{\cdot}39 \times 10^{10} \pm 0{\cdot}18$ $1{\cdot}10 \times 10^{10} \pm 0{\cdot}16$	4·1	$1{\cdot}57 \times 10^{10} \pm 0{\cdot}24$ $2{\cdot}18 \times 10^{10} \pm 0{\cdot}13$
S. thermophilus + *L. fermenti*	6·3	$5{\cdot}38 \times 10^{6} \pm 0{\cdot}09$ $2{\cdot}29 \times 10^{6} \pm 0{\cdot}04$	4·3	$1{\cdot}56 \times 10^{10} \pm 0{\cdot}19$ $1{\cdot}84 \times 10^{10} \pm 0{\cdot}17$	4·3	$3{\cdot}38 \times 10^{8} \pm 0{\cdot}12$ $2{\cdot}53 \times 10^{9} \pm 0{\cdot}14$
S. thermophilus + *L. fermentum*	6·4	$2{\cdot}36 \times 10^{6} \pm 0{\cdot}32$ $1{\cdot}79 \times 10^{7} \pm 0{\cdot}34$	4·3	$1{\cdot}38 \times 10^{9} \pm 0{\cdot}59$ $7{\cdot}37 \times 10^{9} \pm 0{\cdot}13$	4·2	$1{\cdot}42 \times 10^{9} \pm 0{\cdot}52$ $4{\cdot}03 \times 10^{9} \pm 0{\cdot}56$

All results are the means of two identical experiments.

Table 3. Carbohydrate contents ($mg\,ml^{-1}$) in soya milk (12% TS) after 10 and 24 h fermentation by lactic acid bacteria

	Soya milk (12% TS) carbohydrate contents ($mg\,ml^{-1}$)								
	Initial range			After 10 h			After 24 h		
Micro-organisms	Sucrose	Raffinose	Stachyose	Sucrose	Raffinose	Stachyose	Sucrose	Raffinose	Stachyose
S. thermophilus	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	3·35 ± 0·08	4·87 ± 0·76	3·17 ± 0·26	0·36 ± 0·26	2·81 ± 0·71	2·99 ± 0·28
L. delbrueckii subsp *bulgaricus*	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	8·73 ± 1·20	5·28 ± 0·32	6·43 ± 2·09	6·89 ± 2·29	5·33 ± 2·18	8·50 ± 0·93
L. fermenti	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	17·30 ± 1·00	4·17 ± 0·54	3·03 ± 0·34	21·21 ± 4·69	3·85 ± 0·76	2·02 ± 0·20
L. fermentum	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	3·45 ± 0·17	4·57 ± 0·21	2·49 ± 0·40	3·80 ± 0·69	2·98 ± 1·00	2·70 ± 0·42
S. thermophilus + *L. delbrueckii* subsp *bulgaricus*	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	7·05 ± 0·50	6·44 ± 1·01	4·37 ± 0·68	0·27 ± 0·20	5·04 ± 2·44	3·77 ± 0·85
S. thermophilus + *L. fermenti*	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	7·06 ± 0·35	7·41 ± 0·72	3·74 ± 1·34	0·00 ± 0·00	2·19 ± 0·17	2·68 ± 0·34
S. thermophilus + *L. fermentum*	22·59 ± 0·90	4·94 ± 0·10	8·50 ± 0·42	2·60 ± 0·86	5·40 ± 0·93	3·57 ± 0·85	1·00 ± 0·08	3·72 ± 0·67	2·76 ± 0·74

All results are the means from two identical experiments.

mixed culture were only slightly different from those in the individual cultures and, once again, the drop in pH was more rapid in YGSM (pH 4·1 at 24 h) compared with 4·5 in soya milk. It was notable that these figures broadly correlate with results for *L. delbrueckii* subsp *bulgaricus* alone, so that the obvious synergisism that occurs between these species in bovine milk (Tamime and Robinson 1985) did not manifest itself.

The counts of *S. thermophilus* ($1{\cdot}56 \times 10^{10}$) and *L. fermenti* ($1{\cdot}84 \times 10^{10}$) inoculated as a mixed culture (1:1) were higher in YGSM after the first 10 h than those in soya milk—$1{\cdot}04 \times 10^{9}$ and $8{\cdot}57 \times 10^{8}$, respectively. The *S. thermophilus/L. fermentum* combination produced similar counts in soya milk at 10 h: $9{\cdot}00 \times 10^{9}$ and $4{\cdot}48 \times 10^{9}$, respectively, but in this case, YGSM did not cause any increase. Again in YGSM, both combinations achieved pH values of 4·3 in 10 h, but while *S. thermophilus/L. fermentum* reduced the pH of soya milk to 4·7 in 10 h, *S. thermophilus* and *L. fermenti* only generated sufficient acid to reach 5·1 in the same time.

Overall, it would appear that the addition of glucose and yeast extract to soya milk did produce a marked stimulation in the rate of acid production by the selected cultures. The final pH values were reduced also in YGSM, and it may be that, for some purposes, enrichment of the soya milk will be necessary to achieve a pH ~ 4·5 (the coagulation point of soya protein) within an acceptable time-frame. In addition, it was notable that the performance of cultures expected to be oligosaccharide-positive, e.g. *L. fermentum*, did not differ appreciably from the oligosaccharide-negative *L. delbrueckii* subsp *bulgaricus*, suggesting that other carbohydrates are utilized in preference to oligosaccharides.

Utilization of oligosaccharides

The principal oligosaccharides in soya milk, raffinose and stachyose, are galactooligosaccharides that can be hydrolysed by the enzyme, α-galactosidase. The α-galactosidases from lactobacilli are active between pH 4·5–8·0, but exhibit pH optima in the rather narrower range of 5·2–5·9 (Mital et al. 1973).

The quantification of the oligosaccharides, together with the main disaccharide, sucrose, was achieved by comparison of peak heights with standards of sucrose, raffinose (Merk Ltd., Lutterworth, UK) and stachyose (Sigma-Aldrich Company Ltd., Poole, UK). With the exception of *L. fermenti* (see Table 3), all the lactic acid bacteria tested could utilize sucrose in soya milk. *S. thermophilus* exploited this substrate more efficiently than the lactobacilli, and the substantial acid production in soya milk confirms the findings of Mital and Steinkraus (1975). Among the lactobacilli, the metabolism of sucrose by *L. fermentum* was most active over the first 10 h (see Table 3), but in the mixed cultures, the pattern was affected by the presence of *Streptococcus*.

The comparative values for the utilization of stachyose over the 24-h period were: *S. thermophilus*, 64·8% of the original concentration was metabolized, *L. delbruecki* subsp *bulgaricus*, 0·0%, *L. fermenti*, 76·2% and *L. fermentum*, 70·4%, while the mixed cultures showed the following pattern—*S. thermophilus* and *L. fermenti*, 69·6%, *S. thermophilus* and *L. fermentum*, 68·4%, and *S. thermophilus* and *L. delbruecki* subsp *bulgaricus*, 55·7%. This breakdown may well involve hydrolysis of stachyose to galactobiose and sucrose or, alternatively, to manninotriose and fructose, with both the sucrose or fructose being readily utilized by the cultures. Whether or not this reduction in stachyose concentration would have any nutritional significance is a matter for speculation.

The raffinose contents in the soya milks also decreased over the period of fermentation, except in the presence of *L. delbruecki* subsp *bulgaricus*. The hydrolysis of raffinose to melibiose and fructose could be one pathway involved (Liener 1981, Meredith et al. 1988), and it may be that *L. delbruecki* subsp *bulgaricus* masked any such degradation by synthesizing raffinose from sucrose and galactose through the mediation of α-galactosidase. If raffinose has the same flatu-

lence-inducing properties as stachyose, then clearly any reduction in concentration will be a benefit, but only the combination of *S. thermophilus* and *L. fermenti* produced a decrease of over 50%.

Nevertheless, the results of this investigation confirmed that lactic acid bacteria can grow well and produce acid in soya milk and, in most cases, utilize at least one of the oligosaccharides which are believed to contribute to the discomfort associated with eating soya beans (Rackis 1981). Since high rates of growth and lactic acid production—at least pH 4·5 (the coagulation point for soya protein) after overnight incubation (16–18 h)—in unfortified soya milk would be considered to be desirable characteristics for a culture to be used in the production of a fermented soya product, the mixed culture of *S. thermophilus* and *L. delbruecki* subsp *bulgaricus* (1:1) would appear to be the most appropriate combination. Thus, while the pairing of *S. thermophilus* and *L. fermenti* generated a broadly comparable pH, an informal assessment of the flavour profiles of the end products indicated that the latter combination gave a fermented milk with a distinct off-flavour compared with the clean, acidic flavour imparted by the yoghurt culture.

It was also evident that *S. thermophilus* was the essential member of any pairing and, in a commercial operation using direct-to-vat cultures, there may be advantages in employing this organism alone.

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Production and properties of a semi-hard cheese made from soya milk

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Summary A semi-hard soya cheese, with mean moisture content 61.5%, crude protein 21.8% and fat 2.6%, was produced from reconstituted soya-milk powder using a starter culture of *Streptococcus thermophilus* and *Lactobacillus fermentum*. The physical properties of the cheese, as determined with a Texture Profile Analyser, were similar to a cheese made to the same compositional standards from bovine milk. A taste panel of Far Eastern subjects did not find the flavour of the fresh soya cheese acceptable but, when cubes of the cheese (1 cm^3) were deep-fried in corn oil, the hedonic rating improved significantly. It is suggested that the cheese could be used as a protein-rich component of a meal, e.g. to replace meat in a stew, or as a 'snack food'.

Keywords Fermention, lactic acid bacteria, soya milk, cheese, texture and flavour.

Introduction

Soya beans are an excellent source of high quality protein that has many uses as human food (Liu, 1997). In Asia, for example, soya beans have been consumed for centuries and, although use is made of soya flours and similar fractions (Lusas & Riaz, 1995), about 90% of soya-bean protein is consumed in the form of soya-bean curd or tofu. However, because of its high moisture content (90–94%), tofu is prone to spoilage, and it is generally prepared and consumed daily; in addition, it has a bland taste.

In order to obtain a longer shelf-life for tofu, Smith (1958) suggested that the curd might provide a suitable base for making other cheeses. This idea was followed-up by Kenkyusho (1965) who made a cheese-like product from soya milk coagulated by *Enterococcus faecalis*, rennet extract and calcium chloride, while Hang & Jackson (1967a, 1967b) made a cheese from soya milk using *Streptococcus thermophilus* as the starter; the latter product had a clean and fresh flavour, but the moisture content (74–77%) was still too high for prolonged storage.

In an attempt to improve the characteristics of the soya-based cheeses, El-Ella (1980) made a hard cheese from soya milk treated with *Lactococcus lactis* sub-sp. *lactis*, an emulsion of ripened 'Ras' cheese and calcium lactate. The resulting curd was treated in the manner of 'Ras' cheese and, although the body and texture of the ripened cheese were acceptable, the colour and flavour were reported to be poor. Incorporating a percentage of bovine milk improved the flavour, texture and colour of the test cheese and, more recently, Rani & Verma (1995) made an acceptable soya cheese from a mixture of soya and bovine milks. Kim *et al.* (1995) used a similar approach to make a low fat Mozzarella-style cheese.

Although there might be advantages in some countries in extending scarce bovine milk with soya milk, in Thailand and other regions where lactose intolerance is a problem, the production of a hard/semi-hard cheese from soya milk alone could be of benefit. Consequently, it was decided to investigate the possibility of making a semi-hard cheese by fer-

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menting soya milk with *Streptococcus thermophilus* and *Lactobacillus fermentum*, and then processing the curd in the manner described by Robinson & Wilbey (1998) for white-brined cheese. In order to obtain some measure of the success or otherwise of the trial, the chemical and physical properties of the soya cheese were compared with those of both a White Stilton cheese and a 'control' cheese made in the manner of the soya cheese but from bovine milk. However, as the taste and flavour of the soya cheese were not expected to match those of the normal cheeses, the sensory evaluation was confined to the soya cheese.

Materials and methods

Preparation of soya milk

Low-fat, spray-dried soya milk powder supplied from Allergycare (Pollards Yard, Taunton, UK) was used as the base material, and it was dissolved at a rate of 120 g L^{-1} in water deionized by reverse osmosis. After mixing with a high-speed emulsifier/mixer (Silverson Machines Ltd., Waterside, Chesham, UK), the required volume, 5 litres per cheese, was transferred to a stainless steel container with a close-fitting lid.

Propagation of the starter culture

The selection of the culture was based upon some preliminary studies that showed that this specific combination gave satisfactory growth, acid production and utilization of oligosaccharides in soya milk (Chumchuere & Robinson, 1999). A culture of *Strep. thermophilus* NCDO 1496 was obtained from the Dairy Microbiology Laboratory, Department of Food Science and Technology, University of Reading, UK and the culture of *Lb. fermentum* NCDO 1750 was provided by the National Collection of Food Bacteria, Aberdeen, Scotland.

After resuscitation of the freeze-dried cultures, *Strep. thermophilus* was grown in M17 broth and *Lb. fermentum* in MRS broth; these cultures were incubated at 37 °C for 24 h and then kept at 4 °C. The cultures for cheese making were sub-cultured weekly by inoculating 1% (v/v) of an existing, single species culture into 100 mL of sterilized soya milk and incubating at 37 °C for 12–16 h. The resultant culture was used immediately or kept at 4 °C for later use and/or routine transfer.

Procedure for cheese making

Five litres of soya milk (12% TS) were heat treated at 63 °C for 30 min in the stainless-steel container, and then cooled to about 37 °C. Individual cultures of *Str. thermophilus* and *Lb. fermentum* were each added at a rate of 2.5% and, after thorough stirring, the milk was incubated at 37 °C overnight (16 h). By this time, the pH had dropped to about 4.5, and the jelly-like curd was then cut with a knife into pieces of approximately 1 cm^3. After cutting, a slight shrinkage/hardening of the curd was achieved by placing the container in a water bath at 70 °C and holding for 1 h, preliminary trials confirmed that this time–temperature combination produced the optimum degree of coagulation of the soya proteins. The effect of stirring 100 g of calcium sulphate into the curd–whey mixture to assist with the firming of the curd pieces was investigated also (Fukushima, 1980; Wang, 1985).

Although the conventional procedure for making white-brined cheeses involves the transfer of the soft curd to moulds for draining and pressing, it was found that the soya curd retained above 70% moisture even after pressing at 0.6 MPa. Consequently, the traditional system used for Cream cheese (Robinson & Wilbey, 1998) or concentrated yoghurt (labneh) (Tamime & Robinson, 1999) was used for initial dehydration of the curd. This process involved ladling the heat-treated curd into a bag made of a double layer of cheese cloth, and then hanging the bag at 4 °C overnight. In an attempt to assist this drainage of the whey, a further trial was completed in which dry salt (2% NaCl, w/w) was sprinkled onto the curd as it was transferred to the cloth bag.

In all cases, the partly-drained curd was, on the following morning, tipped into a stainless-steel cheese-mould (30 × 10 cm) and pressed for 12 h; the pressure was gradually increased by 0.1 MPa every 45 min until the final pressure of 0.6 MPa was attained. On removal from the mould, the cheese was shrink-wrapped and stored at 4 °C until required for physical, chemical and sensory analysis.

For comparison, a cows' milk cheese was made employing the same basic procedure as that derived for the soya cheese. A commercial sample of a semi-

hard cheese designated as White Stilton provided an additional control sample.

Physical and chemical analysis of soya-milk powder and the cheeses

The moisture and total solids of samples of soya powder and the three cheeses were determined according to ADAS (1986), as was the ash content. The fat contents were measured by extraction with petroleum spirit in a Soxtec extractor (ADAS, 1986), and the total nitrogen contents of the samples were found by the Dumas method using a Leco Nitrogen Determinator (Leco Instruments Ltd., Stockport, UK); the protein content was calculated as total nitrogen multiplied by 6.25.

The physical properties of the cheeses with respect to hardness, cohesiveness, springiness, adhesiveness, gumminess and fracturability (brittleness) were determined with the Texture Profile Analyser (Blackdown Rural Industries, Haslemere, Surrey, UK).

Sensory analysis

As soya products are widely consumed in the Far East, a taste panel of 14 post-graduate students from Thailand and Indonesia was established. The panel was asked to evaluate samples of a one-week old cheese for flavour, texture, colour and appearance using the method of Quantitative Descriptive Analysis (Stone & Sidel, 1985; Powers, 1988). The list of attributes selected by the authors as appropriate for defining the quality of the cheese is shown in Table 2. A linear scale (100 mm) was used, and the terms were anchored with a nil level for any given attribute on the left margin and the extreme level to the right. The distances from the left-hand margin (zero) to the scored points on the scale were then measured, and the mean scores for each attribute reported.

Results and discussion

The chemical composition of the soya-milk powder (means of five replicates) was crude protein 49.3%, water-soluble carbohydrates 9.4%, starch 1.0%, fat 9.2%, ash 5.1% and moisture 3.1%, so that the approximate composition of the 12% suspension was 5.9% crude protein, 1.1% fat and 1.13% soluble carbohydrates. It was established by HPLC (data not shown) that the major sugars were sucrose (5% of the total water-soluble carbohydrates), raffinose (1.1%) and stachyose (3.8%).

The analysis of the soya cheese (means of duplicate samples from five separate cheeses ± standard errors) was moisture 61.5% ± 0.9, crude protein 21.8% ± 0.2 and fat 2.6% ± 0.3; the moisture content of the cows' milk cheese (control) was 59.5% ± 0.5, and that of the White Stilton was 44.8% ± 0.2.

The physical properties, as measured with the Texture Profile Analyser, are shown in Table 1, and interpretation of the profiles was based on the suggestions of Brennan (1980). In particular, it should be noted that the hardness and fracturability of the soya cheese gave values of 28.5 and 27.0 N, respectively, compared with 36.3 and 28.5 for the test cheese made with cows' milk; these differences were not significant ($P > 0.05$). However, although the White Stilton had a lower moisture content, it was softer than both the experimental cheeses, and crumbled much more readily. Clearly any resemblance to the soya cheese was totally superficial, and no attempt was made to match the characteristics of the soya cheese with those of the White Stilton.

In cheeses like Cheddar, sodium chloride is employed to control moisture content and texture but,

Table 1 Physical characteristics of two soya cheeses (see text for addition of $CaSO_4$) and a cows' milk cheese made by hanging the curd overnight at 4 °C in a cloth bag and then pressing the drained curd in a mould at 0.06 MPa; all figures are the means of twenty measurements made for two separate cheeses. The commercial White Stilton was included for comparison

Sample	Hardness (N)	Cohesiveness	Springiness	Fracturability (N)	Gumminess
Soya cheese	28.5^{a}	0.2	0.6^{b}	27.0^{b}	4.5^{a}
Soya cheese + $CaSO_4$	29.9^{a}	0.1	0.6^{b}	30.5^{b}	4.1^{a}
Cows' milk	36.3^{a}	0.2	0.7^{b}	28.5^{b}	3.7^{a}
White Stilton	21.1^{b}	0.1	0.4^{a}	17.1^{a}	2.4^{a}

Differences between the samples were determined using Duncan's multiple range tests at the 0.05 level – samples with the same superscript letter within a column are not significantly different.

Table 2 Mean scores for selected sensory attributes of fresh soya cheese and for portions of the same cheese cooked in corn oil

Attribute	Fresh soya cheese	Fried soya cheese
Colour Intensity	43.6	85.1****
Body and Texture		
Firmness	73.7	83.1****
Open texture	26.6	42.3*
Taste		
Acidity	53.4	54.6
Salty	17.3	19.9
Bitterness	19.0	19.6
Astringency	48.9	34.1**
Flavour		
Strong	32.1	47.3**
Cheesy	24.0	17.9
Fermented	43.3	31.5*
Beany	59.6	45.7**
Rancid	14.2	24.5*
Hedonic Rating	33.5	54.1***

Significant differences between the samples were determined using Duncan's multiple range tests at: *, 0.1 , **, 0.5, ***, 0.01 and ****, 0.001 levels, samples within a row without superscripts have similar properties.

in the present context, the addition of NaCl (2%) to the curd in the bag lead to a decrease in hardness, cohesiveness and fracturability of the resultant cheese; the moisture content remained unchanged. This effect can be explained by the fact that the native soya-protein molecules unfold during heating, so exposing numerous free SH groups, disulphide bonds and hydrophobic groups. If these groups can be linked through neutralization of the negative charges, irreversible aggregates result, but this irreversible coagulation needs an ion with two positive charges that can bind to both negatively-charged amino acid residues and the sulphide groups of the unfolded protein molecules (Fukushima, 1981); it can be assumed that univalent sodium ions simply neutralize the negative charges but cannot forge links between the molecules.

However, the salt that is generally used to coagulate soya milk in tofu making, namely $CaSO_4$ (Fukushima, 1981), does possess the necessary divalent ions, and the addition of $CaSO_4$ lead to both a reduction in moisture content and an increase in hardness of the soya cheese; the moisture content was reduced to 60.5%, and the values for hardness and fracturability rose to 29.9 and 30.5 N, respectively (see Table 1). However, even though the additional calcium might offer a nutritional benefit, the physical differences induced by the salt were not significant ($P > 0.05$).

The results of the sensory analysis are given in Table 2. The hedonic rating (dislike extremely like^{-1} extremely) was only 33.5 out of a possible 100 for the fresh soya cheese. It is likely that certain flavour notes like 'beany', 'astringent' and 'acidic' helped to depress the hedonic score, while the culture did not produce any metabolites capable of masking the undesirable components of the profile. The addition of a strong, artificial flavour might have proved helpful but, instead, it was decided to explore the possibility of using the soya cheese as a component of a meal.

Consequently, a typical soya-milk cheese was cut into cubes (1 cm dimensions), and the cubes were either added to a stew in place of meat (informal assessment only) or was deep fried in corn oil. When the same taste panel was served with individual portions of the deep-fried cheese, the mean hedonic score improved significantly to 54.1. The descriptive attribute responses of the panel are shown in Table 2, and it is noticeable that cooking improved both firmness and texture, and that some of the less desirable features associated with soya beans, e.g. astringency and 'beany' flavour, were reduced in intensity. Differences arising from the cooking process, such as enhanced colour and overall flavour (strong) were not unexpected, but the changes did not appear to prompt any adverse reactions.

Given that the soya cheese could prove to be an acceptable source of dietary protein and fat, it was disappointing that: (a) the moisture content of the test cheeses could not be reduced below 60%, for this level would leave the product susceptible to mould spoilage especially at the ambient temperatures likely to be encountered in Asia; and (b) the response of the taste panel to the fresh cheese was not more positive. Nevertheless, portions of the cheese were acceptable as deep-fried 'snacks', and the use of the material as a meat-substitute in stews remains a possibility. In addition, the low pH of the cheese (mean of 4.4) should mean that any spore-formers present in the original milk will not germinate during storage of a cheese at 4 °C, while any coliforms or similar micro-organisms acquired as post-heating contaminants should decline in numbers ahead of handling in the kitchen and/or consumption.

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The taste test

UK food technologists ran a trial to see if consumers could distinguish between raw and pasteurised milk cheeses. By Karen Nicol and Richard Robinson*

Pasteurised or unpasteurised? Can consumers really tell the difference?

Over the years, a number of incidents of food-borne infection have been linked with the consumption of cheese made from raw bovine milk (1). In spite of this, sales of cheeses produced from non-pasteurised milk continue to increase.

It is also well known that top quality batches of many cheese varieties, such as Cheddar, can be made from milk that has been heat treated to eliminate all vegetative pathogens. So why do people still insist on consuming raw milk cheeses?

Complex flavour profile

The usual answer to this question is that cheeses made with raw milk have more complex flavour profiles than their pasteurised counterparts. In this way, it is suggested, they give the consumer more pleasure (2,3).

However, objective support for such sensory reactions is rarely available, although it is accepted that a number of primary biochemical pathways are implicated in the development of flavour compounds in cheese (4).

It is also accepted that the operation of these pathways depends on the presence of enzymes associated with the types of bacteria found in raw milk. It is agreed too that significant concentrations of fatty acids are desirable in cheeses like Roquefort or Parmesan (5), and that the release of amino acids can contribute to flavour development (6).

Equally important is the release of the many secondary metabolites that can, given suitable conditions of maturation, enhance the complexity of a flavour, eg the floral note associated with the synthesis of phenylethanol in some cheeses (7).

However, the very derivation of these essential flavour profiles implies that the components of the milk have been subject to degradation by enzymes of microbial origin. Clearly, many of the non-starter bacteria responsible for the biochemical changes are harmless saprophytes, but the fact remains that they must be present in the base milk in high numbers if they are to have any impact.

For example, detectable lipase activity in raw milk suggests a colony count of *Pseudomonas* or similar genera of over 1.0×10^7 colony-forming units (cfu) per ml (5). Meanwhile, the non-starter population of lactic acid bacteria in raw milk cheeses can rise above 1.0×10^8 cfu/ml during maturation (8).

Clearly these relatively harmless bacteria are generating compounds perceived by many consumers as essential, but the sheer numbers must raise the spectre of pathogens being present as well.

For cheeses like Parmesan, which are matured for 12 months or more, contaminant pathogens are of less concern, as conditions within the cheese tend to become increasingly inhospitable with the passage of time. The same conclusion does not apply, however, to hard-pressed or semi-hard cheeses which may be consumed within six months of production.

Producers of such cheeses from raw milk may argue that good quality raw milk does not pose a significant risk as total viable counts of bacteria should not exceed 10,000 cfu/ml. But, if that is the case, will there be enough microbial activity of non-starter origin to generate a distinctive flavour?

Put to the test

Given this apparent contradiction, it was decided to produce semi-hard cheeses from both good quality raw milk and pasteurised milk, and then to determine by means of a taste panel whether there was any difference in flavour between the cheeses. The entire trial was carried out twice.

Sufficient raw milk (90 litres) for each trial was obtained from the University of Reading Farm, with typical total colony counts of less than 10,000 cfu/ml. One batch of milk (45 litres) was then pasteurised at 72°C for 15 seconds and cooled to 32°C. The other batch was warmed to 32°C only.

Beyond this point, both vats of milk were treated in an identical fashion, in that the milk was subject to the following steps (9).

- Inoculation with a culture consisting of *Lactococcus lactis* subsp *lactis* and *Lactococcus lactis* subsp *cremoris*.
- Ripening for 45 minutes prior to the addition of rennet.
- Coagulation over 60 minutes until ready for cutting.
- Scalding by increasing the temperature to 36°C over 50 minutes.
- Draining of the whey at pH 6.1.
- Piling of the curd along the sides of the vat.
- Cutting and draining of the blocks of curd until the pH of the whey was 5.5
- Milling and salting (2% w/w) of the curd.
- Moulding and application of minimum pressure to encourage whey drainage.
- Increasing pressure to 0.5 MPa over the next hour and leaving overnight.
- Vacuum packing, cooling to 2°C and holding for five days.
- Maturation at 10°C for 12 weeks.

At the end of the maturation period, duplicate samples of 100g were removed from each cheese with a trier and analysed for *Listeria monocytogenes* (10), *Salmonella* spp (11) and faecal coliforms (11). Additional samples were then taken for analysis for pH, moisture, sodium chloride, crude protein and total fat (12, 13).

However, potential consumer reaction was taken as the central issue, and the following aspects were assessed.

Texture – Cubes of cheese ($1cm^3$) were placed, in turn, under the probe of the General Food Texturometer (Instron, High Wycombe, UK). The

Table 1. Chemical compositions of raw and pasteurised milk cheeses

	Raw milk cheese	Pasteurised milk cheese
pH	4.7	4.7
Fat (%)	32.9	31.6
Crude protein (%)	26.2	22.9
Moisture (%)	35.4	40.9

Table 2. Physical characteristics, as measured by the Instron Texturometer

	Raw Milk Cheese	Pasteurised milk cheese
Springiness	0.65	0.65
Cohesiveness	0.43	0.31
Chewiness	19.0	7.04
Adhesiveness	-1.38	-0.71
Hardness	67.54 N	34.99 N

resistance of the cheese to deformation gives force/time values which can be interpreted in terms of hardness, cohesiveness, chewiness, springiness and adhesiveness (14).

Sensory attributes – A list of attributes covering appearance (white/yellow, open/close texture), odour (intensity of 'cheesy' and lactic aromas), texture (soft/firm, crumbly/coherent) and flavour (cheesy, lactic, creamy, persistent) was compiled from qualities proposed by Muir and Hunter (15).

Each of 18 panellists was presented with two randomly coded samples under artificial daylight illumination in an isolated, air-conditioned booth. A form was provided that listed the above features beside 10cm line-scales with anchor points. Assessors were asked to rate each attribute in turn for one sample. The operation was then repeated for the second sample.

The order of presentation of the samples was randomised to avoid preferences caused by the consumption sequence. The data were analysed by Multiple Variance Analysis (ANOVA), and the Least Significant Differences were calculated. In addition, each of the tasters was asked which sample of cheese he or she preferred.

Free from pathogens

Both types of cheese were found to be free from the pathogens considered to be possible contaminants, a result that served both to confirm the view that good quality raw milk need not always pose a risk to consumers and to give confidence to the taste panel.

Chemical analyses of the cheeses revealed the results shown in Table 1. As the original milks and the process conditions were – for all practical purposes – identical, the difference in moisture content is likely to have resulted from the influence of heat on the whey/casein fractions of the milk.

In particular, any denaturation of whey proteins and their absorption on the caseins tends to reduce syneresis (16), and it is feasible to suggest that this retention of excess whey gave cheeses of higher moisture content.

From an economic point of view, an additional 50g of water per kilo of cheese could be attractive, but only so long as the consumer is not deterred by any possible changes in texture.

As shown in Table 2, the physical properties of the two cheeses were quite different. In particular, the value for hardness of the raw milk cheese was almost double the figure recorded for the pasteurised milk cheese. The related attribute of chewiness showed a contrast that was almost threefold.

These differences do not imply, of course, that one cheese is better, but the figures do suggest the process for handling pasteurised milk would need to be modified if the end-products were to become, at least in physical terms, directly comparable.

The differences revealed by the texturometer were also apparent to the panellists (Figure 1). The perceived differences for firmness and crumbliness were significant ($P < 0.05$).

The lower moisture content may have been responsible, at least in part, for the firm texture of the raw milk cheese. But the more crumbly texture and visibly fissured appearance ('open' texture) of the pasteurised milk sample suggests that the heat treatment had modified the extent of protein-protein bonding as well.

However, any physical differences did not affect the perceptions of flavour or aroma. As shown in Figure 2, there were no significant differences between the two cheeses for any of the attributes examined. Whether or not a trained panel would have been more discriminatory is an open question, and it might be argued that the use of a more extensive range of attributes might have proved more informative.

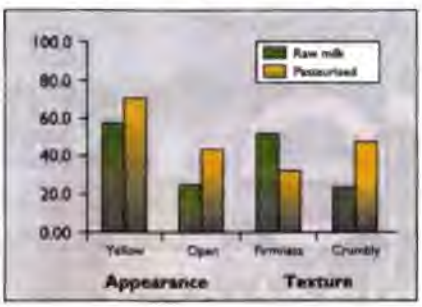

Figure 1. Scores for appearance and texture

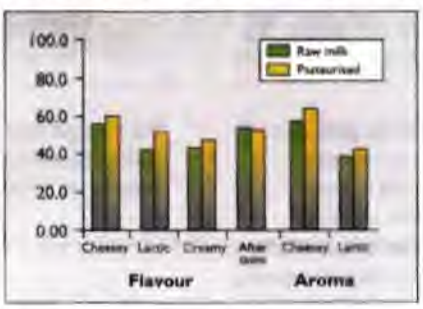

Figure 2. Scores for flavour and aroma

Nevertheless, as the tasters were typical consumers of medium grade, medium price cheeses of the type found in any supermarket, their reactions cannot be dismissed. This is especially true in light of the answers to the question of overall preference. The answers gave a split of ten in favour of the raw milk cheese and eight for the product made from pasteurised milk.

Obviously it is not claimed that the results of this trial are conclusive. But, assuming that the cheesemaking process could have been modified to obtain a better match of the physical characteristics, then it seems the cheeses would, to all intents and purposes, have become indistinguishable.

In other words, it would appear that raw milk cheeses will only become detectable by typical consumers if the microbiological quality is low enough to allow active catabolism of the proteins and fat in the milk, along with the build-up of flavour-enhancing peptides, fatty acids and their derivatives. However, as the numbers and range of bacteria in this 'desirable' microflora increases so, on occasions, will the risk from pathogens occupying the same ecological niches.

It is perhaps curious, therefore, that the amount of raw milk cheeses on supermarket shelves is increasing all the time. This means that either consumers are unaware of or unconcerned about the potential problem of foodborne disease, or there is genuine desire for cheeses with more pronounced and complex flavour profiles.

If this latter point is valid, then rather than expose consumers to the risks associated with raw milk cheeses, perhaps the supermarket chains and major cheesemakers should join forces to raise the quality of cheeses made from pasteurised milk.

Twin targets

Technically, the twin targets of high quality and large volumes of production are not incompatible. Longer maturation times offer an obvious solution, but the additional expense of storage could well prove unacceptable to the major retail chains.

Alternatively, if the non-starter lactic acid bacteria are essential sources of the enzymes associated with flavour production, there is no reason why selected strains of food-grade, flavour-

enhancing lactobacilli should not be added to pasteurised cheesemilk (18).

In a study carried out separately from the one under discussion here – but which also employed a taste panel – *Lactobacillus paracasei* subsp *paracasei* was added to pasteurised milk used for the production of a white brined cheese. It gave an end product that was barely distinguishable from the traditional cheese made with raw milk and no starter culture.

To suit UK territorial varieties, a modification of this system might involve the employment of two cultures, ie acid-producing strains of lactococci for rapid ripening in the vat, and a second culture to enhance flavour production during maturation. Once such a routine has been established, the increase in manufacturing costs is negligible.

The outcome would be the provision of cheeses that: (a) barring a major failure at a critical control point, should be free from vegetative pathogens; and (b) have the desirable flavour characteristics of their raw milk counterparts.

Whether dairies would be willing to adopt this route remains to be seen. But this present study does suggest good quality raw milk does not make cheeses with flavour profiles any different from those identified in similar products made from pasteurised milk. ■

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Putting raw milk cheeses to the test

UK food technologists ran a trial to see if consumers could distinguish between raw and pasteurised milk cheeses. The authors, Karen Nicol and Richard Robinson, are from the University of Reading's Department of Food Science & Technology.

Over the years, a number of incidents of foodborne infection have been linked with the consumption of cheese made from raw bovine milk.[1] In spite of this, sales of cheeses produced from non-pasteurised milk continue to increase.

It is also well known that top-quality batches of many cheese varieties, such as cheddar, can be made from milk that has been heat treated to eliminate all vegetative pathogens. So, why do people still insist on consuming raw milk cheeses?

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Equally important is the release of the many secondary metabolites that can, given suitable conditions of maturation, enhance the complexity of a flavour, e.g. the floral note associated with the synthesis of phenylethanol in some cheeses.[7]

However, the very derivation of these essential flavour profiles implies that the components of the milk have been subject to degradation by enzymes of microbial origin. Many of the non-starter bacteria responsible

for the biochemical changes are harmless saprophytes, but the fact remains that they must be present in the base milk in high numbers if they are to have any impact.

For example, detectable lipase activity in raw milk suggests a colony count of *Pseudomonas* or similar genera of more than 1.0×10^7 colony-forming units (cfu) per mL.[5] Meanwhile, the non-starter population of lactic acid bacteria in raw milk cheeses can rise above 1.0×10^8 cfu/mL during maturation.[8]

Clearly, these relatively harmless bacteria are generating compounds perceived by many consumers as essential, but the sheer numbers must raise the spectre of pathogens being present as well.

For cheeses like parmesan, which are matured for 12 months or more, contaminant pathogens are of less concern, as conditions within the cheese tend to become increasingly inhospitable with time. The same conclusion does not apply, however, to hard-pressed or semi-hard cheeses, which may be consumed within six months of production.

Producers of such cheeses from raw milk may argue that good-quality raw milk does not pose a significant risk, as total viable counts of bacteria should not exceed 10,000 cfu/mL. But, if that is so, will there be enough microbial activity of non-starter origin to generate a distinctive flavour?

Given this apparent contradiction, it was decided to produce semi-hard cheeses from good-quality raw milk and pasteurised milk, and then to determine, by means of a taste panel, whether there was any difference in flavour between the cheeses. The entire trial was carried out twice.

Sufficient raw milk (90 litres) for each trial was obtained from the University of Reading Farm, with typical total colony counts of less than 10,000 cfu/mL. One batch of milk (45 litres) was then pasteurised at 72°C for 15 seconds and cooled to 32°C. The other batch was warmed to 32°C only.

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- maturation at 10°C for 12 weeks.

At the end of the maturation period, duplicate samples of 100g were removed from each cheese with a trier and analysed for *Listeria monocytogenes*,[10] *Salmonella* spp[11] and faecal coliforms.[11] Additional samples were taken for analysis for pH, moisture, sodium chloride, crude protein and total fat.[12,13]

Potential consumer reaction was taken as the central issue and the following aspects were assessed.

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Free from pathogens

Both types of cheese were found to be free from the pathogens considered to be possible contaminants, a result that served both to confirm the view that good-quality raw milk need not always pose a risk to consumers and to give confidence to the taste panel.

Results of the chemical analyses of the cheeses are shown in Table 1. As the original milks and the process conditions were – for all practical purposes – identical, the difference in moisture content is likely to have resulted from the influence of heat on the whey/casein fractions of the milk.

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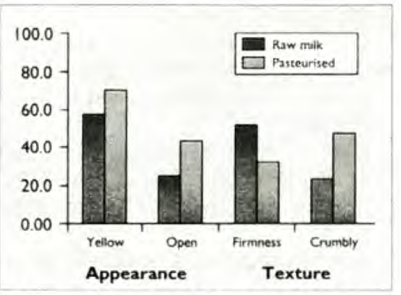

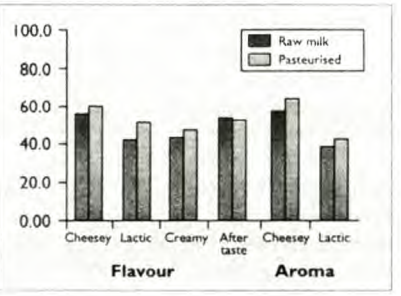

● **Figure 1: Scores for appearance and texture (above). Figure 2: Scores for flavour and aroma (below). Bars at left of pairs represent raw milk cheeses, bars at right pasteurised milk cheeses.**

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If the latter is valid, then rather than expose consumers to the risks associated with raw milk cheeses, perhaps the supermarket chains and major cheesemakers should join forces to raise the quality of cheeses made from pasteurised milk.

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This article was originally published in the the European journal, 'Dairy Industries International', August 1999, and is reprinted with the permission of the editor and authors. *Karen Nicol and Richard Robinson, can be contacted by phone +44 118 931 8700, fax +44 118 931 0080 or e-mail food@afnovell.reading.ac.uk*

R. K. Robinson

A method for the objective assessment of cheese flavour

THE view that foodstuffs should be graded according to quality was at one time accepted as normal practice, but as retail sales have grown in the direction of convenience foods and/or packaging, so consumer attitudes have been conditioned to accept mediocrity as the "norm".

It can be argued, of course, that improvements in quality must involve higher prices but, for a product like Cheddar cheese, this reasoning appears somewhat suspect. Thus, technical innovations over the last 20 years have raised productivity to an unprecedented degree so that, assuming the process is being effectively controlled, there is no reason why a product that is both competitively priced and of top quality should not be made available to the consumer. The validity of this view is supported by the fact that some factories consistently market cheese that cannot be faulted for texture or flavour, and if high standards of quality can be achieved at one plant, then why cannot such standards be accepted universally?

One procedure that could assist in removing low grade cheese from the retail market would be a National Grading Scheme along the lines employed in Scotland, and a similar scheme was, of course, operated in England for many years. Advocates of this approach argue that if all home-produced and imported cheese were to be subjected to an objective scrutiny, then the housewife could be assured that a given grade would be of a specified quality. The advantages of a grading scheme seem, as far as the consumer is concerned, to be self-evident, but the proposal is opposed on the grounds that:

(i) "in-house" schemes run by the major producers have rendered a national approach unnecessary;
(ii) the advantages would not justify the additional cost;
(iii) that skilled personnel to carry out the scheme are not available.

The first point is, of course, a reasonable one, but the fact that blocks of "compressed casein" can appear on the market labelled as "Cheddar cheese" would tend to indicate that a fair percentage of cheese is evading effective scrutiny. The cost of eliminating the observed fluctuations in quality would have to be borne by the consumer, but the advantages of product reliability would more than compensate for any minor increase in price. Implementation of a grading scheme does depend, naturally enough, on the availability of skilled graders, and it is argued that, with existing expertise confined to the major producers, any national approach could not be operated.

This latter view is based on the traditional attitude that cheese grading is essentially an "art" rather than a science, and that the almost intuitive skills of the grader cannot be duplicated by more modern techniques of appraisal. Obviously no one would dispute the ability of a professional grader (Harper, 1956 and 1961), but in the absence of such skill, an alternative approach may have to be sought.

It is the aim of this paper, therefore, to examine one method of quality appraisal to assess whether it could:

(a) be employed to characterise the flavour associated with Cheddar cheese;
(b) provide an objective, quantitative estimate of the relative intensity of cheese flavour;
(c) be capable of operation using untrained personnel.

Thus, if this aspect of quality could be effectively monitored by non-specialists, then a grading scheme becomes a feasible proposition, and the finite reservoir of expertise need no longer be regarded as a limiting factor.

Materials and methods

The general problems associated with organoleptic appraisal have been reviewed elsewhere (Gridgeman, 1967, Amerine *et al*, 1965), and it was decided that, in the present context, the most appropriate method of examination would be to employ a rating system for certain qualities pertinent to cheese. Consequently, a list of 15 terms associated with cheese flavour was drawn up, covering a range of mild, mature and "off-flavours", and the format of the final "score-sheet" is shown in Figure 1. The "off-flavours" ("alternative terms"—Figure 1) were deliberately isolated from the more attractive cheese flavours in order to avoid any distraction of the tasters.

Four samples of cheese were then bought from a local supplier, two being designated as "mild" and two as "mature", and these samples were then cut into small portions and numerically coded. Each taster was offered a "mild" sample and a "mature" sample for examination, and requested to complete the score sheet as instructed; a standard palate cleanser of water and a dry biscuit was taken between samples. Identical pairs of samples were evaluated by 10 tasters chosen at random from the student population, and the second pair of samples was similarly examined by a further panel of 10. Panel size was based on the minimum number of untrained tasters required to identify the essential flavour notes (Harper *et al*, 1968), but with a short period of training, this number might be reduced for certain practical purposes.

Results

The results from the two panels are summarised in Table 1, and it is noticeable that the overall scores assigned to the cheeses are broadly similar. However, if the expected charateristics of Cheddar cheese are grouped according to whether the flavour is associated with "mild" cheese or "mature" cheese, then it is evident that totally inexperienced tasters have little difficulty in characterising a particular sample.

In some respects, it would have been surprising if the scheme had not discriminated between the samples, but what the exercise served to emphasise was that the proposed scheme provided a simple method of quantifying the differences between cheese samples. It was also noted that the pattern of "faults" reflected "maturity" but this association probably indicates merely that some tasters find the

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mature characters too harsh for their liking.

Nevertheless, it is likely that a mature cheese that was really defective would be isolated by the same scheme. It may be relevant also that, assuming the "mature notes" are the main indicators of flavour intensity, then the mature Cheddar has roughly twice the "flavour" score of its mild counterpart, and this comparative feature of the analysis could prove useful in other contexts.

Discussion

The very nature of sensory analysis creates problems for the experimenter, for the choice of terminology is in itself a highly arbitrary facet of the project. Thus, in the present context, some, terms, such as "rich" and "salty", turned out to be be more descriptive than discriminatory, while some tasters felt that "bland" was not a judgment that could be quantified. However, such disagreements are an inevitable part of the evolution of any scheme of quality appraisal, but the important feature of the scheme is that it enabled a panel of untrained personnel to distinguish between different samples of cheese in a manner comparable to that of an expert.

Obviously the number of samples is too small to allow for dogmatic predictions, but the results suggest that designations like "mild" and "mature" are capable of objective, numerical rating. If this view is correct, then it implies that certain types of Cheddar cheese could be expected to generate a specific numerical "profile", and that it would be possible to state that a mild Cheddar (Grade 1) would have to reach a total score (10 assessors) for "mild" characters of $x \pm y$. The establishment of similar targets for other types/grades of hard-pressed cheese could, therefore, make it feasible for a national grading scheme to be introduced irrespective of the availability of skilled graders.

It must be admitted, of course, that texture is an equally important feature of a good cheese, but there is no reason to believe that this feature is not also capable of objective analysis. Thus, Szczesniak (1963), Szczesniak *et al* (1963) and Civille and Liska (1975) have shown that techniques for texture profiling can be applied to a range of food products, and hence it is clearly possible that an "optimum" profile for Cheddar cheese could be derived in the same way.

The erection of a fully operational scheme for cheese grading based on panel assessment would require a great deal more data than has been presented here, but what these results do indicate is that the "art" of the cheese grader is capable of duplication. If this conclusion is correct, then a national grading scheme for hard-pressed cheese can no longer be discounted on the grounds that the necessary expertise is unavailable. Whether the political will can be found to implement such a scheme is open to debate, but at least one argument against the introduction of quality grades for cheese looks less convincing than is sometimes supposed.

Figure 1. The rating system employed to characterise the flavour of samples of Cheddar cheese

Cheese characterisation

The following list of terms have been selected from those often used to describe cheese. You are asked to work through the list with the sample provided, and to indicate the degree or extent to which any of the terms applies to initial flavour or after-taste using the 0-5 scale.,

(0 = none, 5 = strongly.)

Assessor: Date:

Creamy	0	1	2	3	4	5	Sweet	0	1	2	3	4	5
Sharp	0	1	2	3	4	5	Salty	0	1	2	3	4	5
Buttery	0	1	2	3	4	5	Acidic	0	1	2	3	4	5
Tangy	0	1	2	3	4	5	Rich	0	1	2	3	4	5
Bland	0	1	2	3	4	5	Bitter	0	1	2	3	4	5

Alternative terms:

Sweaty	0	1	2	3	4	5
Aromatic	0	1	2	3	4	5
Burning	0	1	2	3	4	5
Sour	0	1	2	3	4	5
Metallic	0	1	2	3	4	5

Sample code:

Table 1. Profile analyses of two types of Cheddar cheese; figures as total scores awarded by 10 assessors. Scores for each panel are recorded separately.

Characteristic	Total score			
	Mild Cheddar		*Mature Cheddar*	
	A	*B*	*A*	*B*
Creamy	25	17	6	5
Rich	22	20	22	16
Buttery	22	14	7	7
Sweet	19	23	7	6
Bland	15	30	1	1
Score for "mild" characters	103	104	43	35
Sharp	9	13	30	23
Bitter	1	7	13	16
Tangy	16	10	29	30
Acidic	17	10	25	20
Salty	17	16	17	23
Score for "mature" characters	60	56	114	112
Sweaty	3	2	5	6
Aromatic	8	13	13	20
Burning	1	1	5	4
Sour	7	3	12	9
Metallic	2	1	4	3
Score for "off-flavours"	21	20	39	42

Footnote. Although this technique can be criticised in that it involves the "addition" of scores for somewhat dissimilar terms, it has been used, in the present context, to "highlight" differences between samples.

Acknowledgments

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NIZO test report on self-desludging separator

At the request of Alfa-Laval NV at Amstelveen, the Netherlands Institute for Dairy Research (NIZO) carried out a series of 18 tests on the skimming efficiency of a newly developed, self-desludging hermetic milk separator, type MRPX 318 HGV, and compared it with that of a second-hand conventional hermetic milk separator, type S 2181 M. Both separators had been manufactured by Alfa-Laval A/B, Sweden.

The tests were carried out under practical conditions. Both separators worked at their nominal capacities. The whole milk was first passed through a plate pasteuriser and after that was divided between the two separators. The milk was obtained from various sources, while some tests were carried out with down-stream pasteurisation and others with up-stream pasteurisation. During each test 11 samples were taken from the skim-milk outlet of each separator during two hours. These samples were mixed, and from this milk two samples were used for the determination in duplicate of the fat content by the Röse-Gottlieb method.

Of the two samples of skim-milk taken from each separator the average fat content was calculated. In 17 out of 18 tests the fat content of the skim-milk from the self-desludging hermetic separator was somewhat lower than that of the skim-milk from the older hermetic separator. These results are related to the given experimental circumstances and the separators used. The skimming efficiency of the self-desludging hermetic separator is, on average, also somewhat better than that of the hermetic separator. The average difference over 18 tests was 0.0073 per cent. This average difference is significant, its standard deviation being 0.00072.

These tests cannot give much information concerning the effect of differences in milk properties and treatment. The results do not seem to be in contradiction with the observation that the skimming efficiency is somewhat better for can milk, which was fresh or approximately 12 hours old in these tests, than for cold-stored milk. This observation is well known. The skimming efficiency decreases after cold storage, which in practice is related to extra physical effects produced in the milk by pumping and stirring.

After the time relay for switching from starting to operation position had been adjusted, the bowl had the correct speed when skimming started; approximately 20 minutes after the motor of the self-desludging hermetic separator had been switched on, the first milk was supplied.

The older hermetic separator was inspected visually after cleaning. The rubber seals were also checked at the same time. These were replaced on some occasions to prevent breakdowns and well before they were likely to be faulty. The test separator was inspected inside visually five times during the test period after cleaning had taken place. In the beginning this was done more frequently than later on. It was observed that after the cleaning programme had been carried out correctly, the discs and the bowl were properly cleaned. Sometimes a few small hard pieces were found in the distribution holes of some upper discs. Probably these had been released during cleaning from places somewhere else in the equipment outside the separator.

About three months after installation of the self-desludging hermetic separator, the axial seals were replaced because the rubber parts of the top seal began to separate from the steel parts, and the seal just below the top seal showed some swelling of the rubber parts. The carbon rings were still in reasonably good condition so that leakage should not have been expected. These seals were replaced after about 1,000 working hours, approximately 300 of which had already elapsed before the seals were installed at the Coberco dairy (as indicated by the manufacturer). During the test period the automation of the cooling system of the separator and its seals was improved. Sometimes in the beginning it was not quite safe in relation to the special test lay-out, so that at some times the seals were without cooling water during starting up or running down. The manufacturer pointed out that all the machines will be provided with a safe and fully automatic system for this section.

Generally the impression was obtained that this new separator can operate in a sufficiently reliable way.—*NIZO report R107.*

New £13m MMB creamery comes on stream

The Milk Marketing Board's latest creamery—Severnside at Stroud in Gloucester—has come on stream. Work began on the £13 million pound project in May 1977 and the first load of milk was processed on 20 March 1979, only 22 months later.

Severnside represents the latest in dairy industry technology and, during peak production will process a maximum of a million litres daily. This represents a daily total of 45 tonnes of butter and 92 tonnes of skim milk powder. The highly mechanised plant will eventually employ 150 staff.

The creamery is part of the Board's overall plan to deal with increasing milk production and when fully operational it will serve milk producers in the west country, South Wales and the south midlands. It will also be of considerable significance in handling the seasonal fluctuations of milk production.

Commenting on the importance of the new creamery, Mr Steve Roberts, MMB chairman, said recently: "Severnside represents a major investment by the Board and is proof of our continuing confidence in the future of the UK dairy industry. It will make a valuable contribution to the work of the Board and the milk producer. The construction of the creamery and its transition from drawing board to commissioned plant in such a short time is a major achievement by main contractors, Emberbrook Engineering Ltd and all those involved".

Annual report on research and development 1977

A report, published in May, on the Ministry of Agriculture's responsibilities for research and development describes, among other things, the progress of the commissioning of research and the conclusions of the reviews of commissioned research. It shows the funds made available by the Ministry during 1977/78 for research and development in agriculture, fisheries and food, and refers to the developments of the national programmes for these areas. Copies of the report can be obtained from HM Stationery Office, price £2.00 (by post £2.12).

The Secretary of State for Employment has appointed Mr Geoff W. Jones as an employer member of the Food Drink and Tobacco Industry Training Board.

Iria ri Matii is a refreshing yogurt-like drink popular in certain parts of Kenya and produced by traditional methods. J M Kimonye and R K Robinson investigate the lactic flora involved and the reasons underlying the method of production

Iria ri Matii – a traditional fermented milk from Kenya

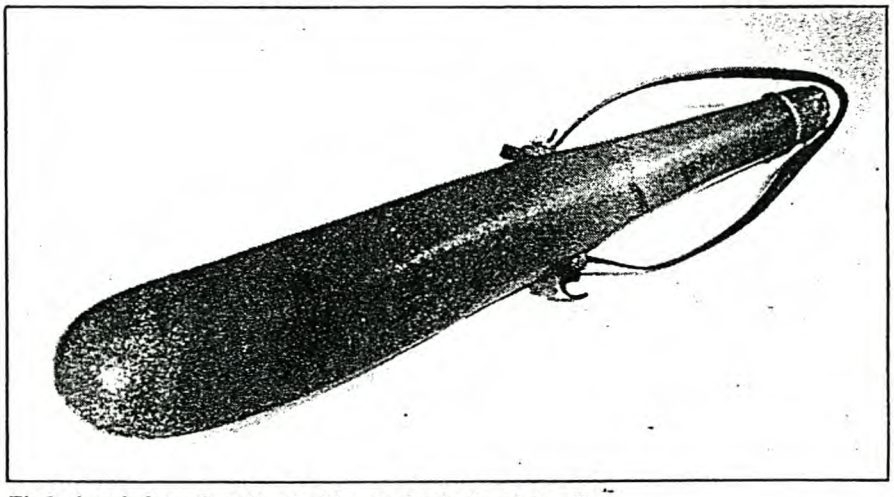

Fig 1: A typical gourd used by the Meru for incubation of the milk.

In rural Africa, the microbiological quality of raw milk is poor and it is not surprising therefore, that 'soured' milk products became a normal part of the diet. The constant use of the same vessel, or the addition of fresh milk to an ongoing fermentation, has gradually led to the evolution of a number of locally popular milks (1).

In Kenya, such a method is practised among the Masais, Turkanas, Kalenjins, Somalis and Merus. The Merus live on the Eastern slopes of Mount Kenya and the community comprises about one million people. They are mainly agriculturists who also keep cattle, sheep and goats and while the cattle provide milk and meat, the goats and sheep are reared solely for meat. Like any community that keeps large herds of cattle, there is plenty of surplus milk.

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Traditionally, this surplus is preserved through a lactic acid fermentation process that yields a yogurt-like product known locally as 'Iria ri Matii'. Like many such fermentations, the making procedure follows precise guidelines handed down from one generation to the next, but despite the widespread popularity of the product, the scientific basis for the various steps is poorly understood. In the absence of this knowledge, there is no possibility of expanding production beyond the present limits; hence the aim of this study was to:

i) investigate the microbiology of the process;
ii) highlight those aspects of the traditional procedure that are essential for the successful completion of the fermentation, and the derivation of a flavour attractive to the local population.

The traditional process

A gourd is prepared by taking a mature, dried fruit of the plant, *Lagenaria leucantha*, removing the seeds and smoothing the inside thoroughly with glowing splints of one particular tree, the Mutero *(Olea africana)*. The inside of the gourd is carefully scratched with the charcoal from the burning wood and this process is repeated until the inside is smooth and even; the gourd must feel reasonably hot from the outside. After it has been properly sooted, the excess dust from the charcoal is removed gently using a whisk made from a cow's tail. The gourd is now considered ready for use, and is covered and left to cool.

Early lactation milk is then put into the gourd to condition it. This procedure is repeated several times, with allowance between each preparation for a complete fermentation to take place and discard of the product. A re-application of charcoal follows the discard of the final batch. The object of treating the gourd in this way would appear to be to introduce a culture from the raw milk and to absorb it onto the inner wall of the gourd ready for future fermentations.

Milk for the production of a batch of 'Iria ri Matii' may be boiled or unboiled. If raw milk is used, it is cooled immediately after milking and poured directly into the gourd. Otherwise, the milk is heated close to boiling, before cooling, partial removal of the cream and filling into the gourd.

In either case, the full gourd is capped with a special cap of animal-skin and left to stand at ambient temperature for three or four days (see figure 1).

At the end of the incubation period, the coagulum is stirred and decanted as a refreshing drink (titratable acidity around 1.0% lactic acid). The attractive flavour/aroma of wood smoke is an essential characteristic of the product. After consumption, the gourd may be refilled immediately and the fermentation repeated, but if there is to be a delay between consecutive batches, then the gourd is rinsed out and dried; re-treatment with hot charcoal is required immediately before the next time it is used.

Microbiology of the process

Although a detailed study of the 'charcoal' process has not been made, the most likely scenario is that certain components of the smoke serve to inactivate potential spoilage organisms in the milk, so allowing lactic acid bacteria from the wall of the gourd to become the dominant flora.

Whether the lactic flora has acquired resistance to the volatiles in the smoke, or whether the cells are merely protected within the walls of the gourd is a matter for speculation, but either way, the routine procedure does lead to a seemingly well-controlled fermentation.

The validity of this view was confirmed, to some extent, by an examination of the 'lactic flora' of traditional products employing Rogosa Agar to enumerate lactobacilli, and Neutral Red Chalk Lactose Agar for streptococci(2). Although no lactobacilli were isolated, streptococci were found on three separate occasions, and at levels of 57×10^6 to 32×10^8 per ml of end-product. The difference between the counts is probably a reflection of variations in incubation time/initial count within the gourd, but the counts do indicate that, at least for the milks examined, streptococci are the dominant organisms.

Counts for yeasts and moulds (Malt Extract Agar at pH 4.5(3)) were >10 cfu/ml for all three samples.

A tentative examination of colonies isolated from the milk suggested that the dominant organism is broadly similar in character to *Streptococcus thermophilus*(4). The thermophilic nature of the organism is not unexpected, because as pointed out by Marshall(5), there is a distinct correlation between climate and the type of microflora found in indigenous fermented milks.

In this particular case, this expected relationship would be reinforced by the heating of the gourd between consecutive batches, because only a distinctly thermoduric species would be likely to survive such a treatment.

Standarisation of the process

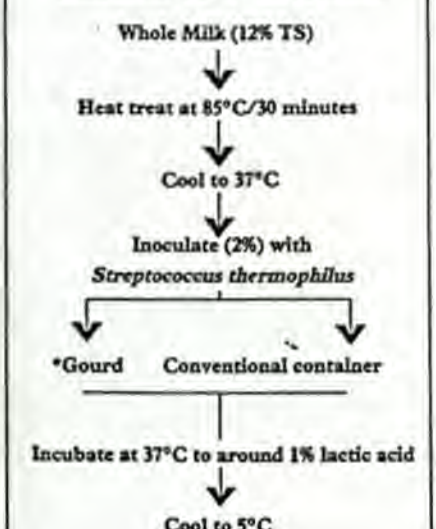
The yogurt-like nature of the end-product and the involvement of thermophilic streptococci as the organisms dominating the fermentation, suggested that a typical process for the production of natural yogurt could be exploited to achieve a standard system for Iria ri Matii. The selected procedure is shown in figure 2.

The traditional route, ie incubation in a pre-smoked gourd, was introduced so that a typical sample of Iria ri Matii would be available for comparison with both the experimental batch and a local batch acquired in Kenya.

In terms of viscosity, acidity and overall organoleptic acceptability, all three products were indistinguishable to an informal taste panel of food scientists, but the smoked flavour of the milks incubated in the gourds was rated as 'highly desirable'.

It would be fair to conclude, therefore, that the basic product could be manufactured on a commercial-scale without difficulty, but matching the traditional flavour may prove more difficult. Some authorities have argued that urban communities are unlikely to appreciate the 'smoked' flavour anyway(6). However, the validity of the comment must be open to debate, as there is no evidence that the products tested by Shalo and Hansen(6) had the authentic taste acquired from Mutero.

Figure 2. Flow diagram of the proposed process for the manufacture of Iria ri Matii



*Gourd product would have the flavour of Mutero Based on Tamime & Robinson (1985).

Discussion

It is clear that the traditional procedure for making Iria ri Matii gives rise to a pleasant flavoured, fermented milk and that the fermentation is dominated by streptococci. The precise role of the 'smoking' with wood of the wild olive has yet to be established but the evidence suggests that components of the smoke suppress naturally-occuring yeasts and moulds, as well as bacterial contaminants brought in by the raw milk.

This elimination of competition enables the streptococci embedded in the wall of the gourd to develop strongly and so give rise to the desired end-product, even though the usual incubation temperature — around 25° C — is well below the optimum for the dominant species.

This view is further supported by comments from the Meru themselves in that if (a) a 'rested' gourd is not charcoal-treated prior to re-use, or (b) treated with charcoal from a different tree, then the product fails to develop correctly and is usually heavily contaminated with yeasts. Obviously, there are no experimental data to authenticate these comments but it does appear that the fermentation is yet another example of a traditional food process that, as a result of astute manipulation of conditions, has evolved into a highly successful technique for preserving an otherwise fragile foodstuff — milk. The thermophilic character of the dominant microflora suggests that the basic fermentation stage could be scaled-up without difficulty, but matching the essential 'smoked' flavour of the end-product may not be so easy.

Nevertheless, products are available for imparting a 'smoked' flavour and aroma to foods and such materials could provide a route for producing Iria ri Matii on a commercial scale.

Acknowledgement

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BREW + BEV TECH '91

Brew + Bev Tech, an International exhibition of drinks and drinks technology is a new event taking the place of BREWEX and BREW. It will be staged at the National Exhibition Centre, Birmingham on 10-13 September 1991.

Dairy Industries International asked Wilf Hipkins, chairman of Brewing Technology Services what benefits Brew + Bev Tech will have for the dairy industry.

Dairy Industries International: Why do you think that there is a need, particularly from the dairy industry point of view, for another exhibition?

Wilf Hipkins: "The drinks' industry — and the technology it encompases — is a global business. The dairy side of the industry is a major market place for companies selling many kinds of processing and packaging equipment."

"I take the view that BREW + BEV TECH '91 will be an excellent showcase for the dairy industry because there has not been a UK-based exhibition opportunity for the dairy industry for many years.

Dairy Industries International: What is BREW + BEV TECH '91 setting out to ahcieve?

Wilf Hipkins: "Its objectives are to provide a UK-based exhibition for all sectors of the drinks' technology industry and an opportunity for UK companies to do business with potential buyers from the UK, but particularly from overseas countries."

Dairy Industries International: Is the exhibition going to achieve what it is setting out to do?

Wilf Hipkins: "One factor in its favour is that it is being regarded by the industry as the industry's exhibition and is supported by all the major trade associations — the Society of Dairy Technology, Allied Brewery Traders' Association, Institute of Brewing, Incorporated Brewers' Guild and the British Soft Drinks' Association. From the above list you can see the breadth of support from industry areas additional to dairy, with whom there is much overlap.

"One initiative launched to emphasise the importance of the dairy industry at the show is a Dairy Trail. This trail, supported by *Dairy Industries International*, will enable key dairy companies to be clearly and quickly identified by prospective customers.

"We are confident that the exhibition will be a great success."